

THE JOURNAL
OF
PHARMACOLOGY
AND
EXPERIMENTAL THERAPEUTICS

FOUNDED BY JOHN J. ABEL

OFFICIAL PUBLICATION
OF THE AMERICAN SOCIETY FOR PHARMACOLOGY AND
EXPERIMENTAL THERAPEUTICS INCORPORATED

Editorial Board

McKEEN CATTELL, *Managing Editor*

CARL A. DRAGSTEDT

J. K. W. FERGUSON

E. K. MARSHALL, JR.

CARL F. SCHMIDT

A. L. TATUM

ARNOLD D. WELCH

VOLUME 98

1948

BALTIMORE, MARYLAND

Reprinted by permission of The Williams and Wilkins Company, Baltimore, Md.

JOHNSON REPRINT CORPORATION
111 Fifth Avenue, New York 3, New York

Johnson Reprint Company Limited
Berkeley Square House, London, W. 1.

First reprinting, 1963, Johnson Reprint Corporation

CONTENTS

NUMBER 1, MAY, 1948

I. The Intravenous, Subcutaneous and Cutaneous Toxicity of <i>bis</i> (β -Chloroethyl) Sulfide (Mustard Gas) and of Various Derivatives. William P. Anslow, Jr., David A. Karnofsky, B. Val Jager and Homer W. Smith...	1
II. The Effects of Intravenous Injection of Tetra-Ethyl-Ammonium Chloride On the Intra-Arterial Blood Pressure and Other Physiologic Variables in Man. George E. Brown, Jr., Earl H. Wood and Edward H. Lambert.	10
III. Effect of Podophyllin on Transplanted Mouse Tumors. Morris Belkin	18
IV. Toxicological Investigations of Compounds Proposed for Use as Insect Repellents. A. Local and Systemic Effects Following Topical Skin Application. John H. Draize, Elsie Alvarez and Marie F. Whitesell. B. Acute Oral Toxicity. Geoffrey Woodard and E. Conway Hagan. C. Pathological Examination. Arthur A. Nelson....	26
V. Locus of the Adrenergic Blocking Action of Dibenamine. Mark Nickerson and George M. Nomaguchi..	40
VI. The Blocking Action of Magnesium Ion on Sympathetic Ganglia. John B. Stanbury.	52
VII. Comparative Properties of Six Phenethylamines, with Observations on the Nature of Tachyphylaxis. Claude V. Winder, Mona M. Anderson and Herve C. Parke	63
VIII. A Comparative Study of the Intravenous Pigeon and the Intravenous Cat Method in the Assay of Digitalis. Herbert A. Braun and Lehman M. Lusky.	81
IX. Relative Experimental Coronary Vasodilator Potencies and Toxicities of Papaverine, Theophylline, and a Papaverine-Theophylline Molecular Association Compound. Claude V. Winder and Margaret E. Kaiser	86
X. The Effect of Dithiobiuret (DTB) on the Electrolyte and Water Content of Skeletal Muscle, and on Carbohydrate Metabolism. Sam Seifter, David M. Harkness, Edward Muntwyler and Joseph Seifter ..	93
XI. A Toxicologic and Pharmacologic Investigation of Cycloheptenylethyl Barbituric Acid (Medomin). William A. Halbeisen, Charles M. Gruber, Jr. and Charles M. Gruber	101
XII The Curariform Activity of d-N-Methyl-Chondrodendrine and d-O-Methyl-N-Methyl-Chondrodendrine. David F. Marsh, Clark K. Sleeth and Eldon B. Tucker	109
XIII. Pharmacological Properties of Sympathomimetic Diamines. G. Lehmann and Lowell O. Randall	114

NUMBER 2, JUNE, 1948

XIV. George Barclay Wallace, 1874-1948	127
XV The Determination of 3-(Orthotoloxyl)-1,2-Propanediol (Myanesin) in Body Fluids and Tissues, and Its Disappearance from the Blood Following Intravenous Injections in the Dog Elwood Titus, Stanley Ulack and Arthur P. Richardson	129
XVI. A Pyrex Apparatus for the Perfusion of the Coronary Circulation of Mammalian Hearts. Frederick F. Anderson and Bradford N. Craver	135
XVII. A Method for the Quantitative Estimation of Theophylline in Blood and Urine: Application to the Dog. Albert J. Plummer	142
XVIII. The Chronic Oral Toxicity of Chloroquine. O. Garth Fitzhugh, Arthur A. Nelson and Oma L. Holland	147

XIX. Effect of Vitamin A Deficiency on the Toxicity of Strychnine. John F. Suchy and Charles F. Poe ..	153
XX. Ouabagenin. K. K. Chen, Robert C. Anderson and Harold M. Worth...	156
XXI. The effect of Adrenalectomy on Morphine Analgesia in Rats. Frances J. Friend, M. S., and S. C. Harris, Ph.D.	161
XXII. Comparative Effectiveness of Five Antihistaminics vs. Histamine-Induced Spasm in Canine Thiry-Vella Loops. Bradford N. Craver, Anne Cameron and Frederick F. Yonkman ..	168
XXIII. The Influence of Diphenhydramine Hydrochloride and Epinephrine on Glucose Metabolism in the Rabbit. Graham Chen and Isabel G. Clarke ..	175
XXIV. Some Studies on the Pharmacology of Relavin. M. X. Zarrow and W. L. Money ..	180
XXV. Sugar Alcohols. XXVI. Pharmacodynamic Studies of Polyoxyalkylene Derivatives of Hexitol Anhydride Partial Fatty Acid Esters. John C. Krantz, Jr., C. Jelleff Carr, Joseph G. Bird and Sally Cook	188
XXVI. Quantitative Measurement of Uterine Responses Using the Strain Gage Dynamometer, with Notes on the Effect of Anti-Histaminic Drugs on the Rabbit Myometrium. R. M. Reynolds and Irwin H. Kaiser....	196
XXVII. The Effect of Central Nervous System Depressants on the Emptying Time of the Human Stomach. David W. Northrup and Edward J. Van Liere.	208
XXVIII. The Toxicologic and Antihistaminic Properties of N,N'-Dimethyl-N'-Phenyl - N' - (2-Thienylmethyl) Ethylenediamine Hydrochloride (Diatrin). N. Ercoli, R. J. Schachter, W. C. Hueper and M. N. Lewis.	210
XXIX. Niasara, A Digitalis-Like Colombian Arrow Poison. K. Mezey, C. Uribe-Piedrahita, J. Pataki and J. Huertas-Lozano	223
XXX. Cholinesterases of Human Erythrocytes and Plasma and their Inhibition by Antimalarial Drugs. C. I. Wright and Jean C. Sabine	230
XXXI. The Effect of Certain Goitrogenic Drugs on the Absorption of Radioactive Iodine by the Thyroid Gland of Rats and Chicks. I. Collection of Radioiodine by Thyroids Made Goitrous Following Chronic Administration of These Agents. R. W. Rawson, D. A. McGinty, Wendell Peacock, Priscilla Merrill, Mary Wilson and Helen Lockhart	240
XXXII. The Effect of Certain Goitrogenic Drugs on the Absorption of Radioactive Iodine by the Thyroid Gland. II. Collection of Radioiodine by Thyroids of Rats and Chicks Following a Single Injection of These Agents. D. A. McGinty, R. W. Rawson, R. G. Fluharty, Mary Wilson, Charlotte Riddell and Hilda Yee	246

NUMBER 3, JULY, 1948

XXXIII. Reid Hunt, 1870-1948	259
XXXIV. Effects of Amphetamine and Cocaine on Neuromuscular Function. J. V. Luco, C. Eyzaguirre and F. Perez ..	261
XXXV. The Effect of Quantitative and Qualitative Protein Deficiency on Tolerance to Emetine. K. Guggenheim and Edith Buechler ..	273
XXXVI. Tissue Distribution of a Toxicant Following Oral Ingestion of the Gamma Isomer of Benzene Hexachloride by Rats. Edwin P. Laug ..	277
XXXVII. Pharmacologic Comparison of the Optical Isomers of Methadon. C. C. Scott, E. B. Robbins and K. K. Chen ..	282
XXXVIII. The Rodenticidal Activity of Fluoroacetphenylhydrazide ("Fanyline") and Its Oral Toxicity to Several Species. With a Note on the Toxicity of 64 Other Candidate Rodenticides. Leonard Karel ..	287
XXXIX. Some Aspects of Thiamine Toxicity. Jay A. Smith, Piero P. Foa, Harriet R. Weinstein, A. S. Ludwig, and J. Marvin Wertheim.....	294

XL. The Addiction Liability of Some Drugs of the Methadon Series. Harris Isbell and Anna J. Eisenman	305
XLl. Pharmacological Studies on Analgesic Piperidine Derivatives. Lowell O. Randall and G. Lehmann	314
XLII. Inhibition of Succinoydase by the Vitamin P-Like Flavonoid 2',3,4-Trihydroxy Chalcone. Grant R. Bartlett	329
XLIII. The Comparative Pharmacology of the Cyclohexylalkylamines. David F. Marsh	338
XLIV. The Effects of Corn Oil and Olive Oil on the Blood Sugar and Rectal Temperature of Rabbits. E. F. Stohlman	346
XLV. The Effect of DDT on the Blood Sugar and of Glucose Administration on the Acute and Chronic Poisoning of DDT in Rabbits. E. F. Stohlman and R. D. Lillie	351
XLVI. The Toxicity and Muscular Effect of d-Tubocurarine Combined with β -Erythroidine, Myanesin or Evipal. F. M. Berger, M.D. and R. P. Schwartz, M.D.	362
XLVII. The Absorption, Distribution, Excretion and Fate of Para-Aminosalicylic Acid. E. Leong Way, Paul K. Smith, Donald L. Howie, Rowena Weiss and Rollan Swanson	368
XLVIII. Effect of Rutin on Coagulation Time of Rat Blood. M. B. Plungian, James C. Munch and J. B. Wolfe	383
XLIX. A Comparative Study of the Activity and Toxicity of Hexylcaine (1-Cyclohexylamino-2-propylbenzoate); a New Local Anesthetic Agent. Karl H. Beyer, Albert R. Latven, Walter A. Freyburger and Mary P. Parker	388
L. The Effect of Sodium Bicarbonate on the Renal Excretion of Salicylate. Florence Williams and Jack R. Leonards	401
LI. Toxicological and Pharmacological Studies on the Powdered Stem of <i>Ryania Speciosa</i> , a Plant Insecticide. Samuel Kuna and Ralph E. Heal	407
LII. The Effect of Various Sulfur-containing Compounds on Alphanaphthylthiourea (ANTU) Toxicity to Rats. Leonard Karel and Bertram J. Meyer	414
LIll. The Effect of Environmental Temperature on Alphanaphthylthiourea (ANTU) Toxicity to Rats. Bertram J. Meyer and Leonard Karel	420
LIV. Comparison of Pressor Action of Alicyclic Derivatives of Aliphatic Amines. Edward E. Swanson and K. K. Chen	423
LV. Chemotherapeutic Studies on a Series of Dithiocarbamates and Their Bismuth Derivatives. Andres Goth and Fabian J. Robinson.	430
LVI. Adrenergic Blocking Drugs. I. Comparisons of Effectiveness in Decreasing Epinephrine Toxicity in Mice. Earl R. Loew and Audrey Micetich	434
LVII. Studies on Nitrate Esters. I. Nitrite-producing Systems in Rabbit Tissues. Fred W. Oberst and Fred H. Snyder	444
LVIII. p-Aminomethylbenzenesulfonamide (Sulfamylon): Its Determination in Biological Fluids, with a Preliminary Report on the Blood Serum Levels Obtained upon Oral and Subcutaneous Administration. M. Lawrence Heideman, Jr., A. B., and Robert C. Rutledge, Jr., M.D.	451
LIX. Dynamics of Recovery and Measure of Drug Antagonism. Inhibition of Smooth Muscle by Lysocithin and Antihistaminics. M. Rocha E. Silva and Wilson T. Beraldo.	457
LX. The Relationship between Chemical Structure and Central Depressant Action of α -Substituted Ethers of Glycerol. F. M. Berger	470
LXI. The Effects of Ether on the Myocardium of the Dog. Leslie L. Bennett and Carl W. Fisher	482
LXII. Index.	487

THE INTRAVENOUS, SUBCUTANEOUS AND CUTANEOUS TOXICITY OF *bis*(β -CHLOROETHYL) SULFIDE (MUSTARD GAS) AND OF VARIOUS DERIVATIVES¹

WILLIAM P. ANSLOW, JR., DAVID A. KARNOFSKY, B. VAL JAGER
AND HOMER W. SMITH

Department of Physiology, New York University College of Medicine, New York

Received for publication July 11, 1947

Mustard gas (*bis*(β -chloroethyl) sulfide) was the major chemical agent used in World War I and it was anticipated in event of the outbreak of chemical warfare in World War II that it would be introduced both as an offensive and defensive weapon. Numerous studies of the toxicological action of this compound were carried out from 1917 to 1939, and it was known that in sufficient doses it induces systemic intoxication which culminates in death (1). However, the mechanism of this systemic intoxication was obscure and it was believed that by the study of related compounds the action of mustard and of other β -chloroethyl vesicants could be correlated with chemical structure and reactivity. The studies reported here were directed towards these objectives. The present paper deals only with toxicity and gross pharmacologic action, data on clinical and microscopic pathology being reported elsewhere (2).

EXPERIMENTAL

Samples of *bis*(β -chloroethyl) sulfide were obtained from the University of Chicago Toxicity Laboratory. β -chloroethyl- β -hydroxyethyl sulfide was synthesized according to the method of Grant and Kinsey (3) from thioglycol and thionyl chloride. β -chloroethyl β -(*bis*(β -hydroxyethyl)sulfonium)ethyl sulfide picrylsulfonate was isolated from an hydrolysate of *bis*(β -chloroethyl) sulfide according to the method of Bergmann and his collaborators (4). Grateful acknowledgment is made to the late Dr. Bergmann and his collaborators for samples of the following: β -chloroethyl β -(*bis*(β -hydroxyethyl) sulfonium)ethyl sulfide picrylsulfonate, β -*bis*(*bis*(β -hydroxyethyl)sulfonium-ethyl) sulfide dipicrylsulfonate, β -hydroxyethyl β -(*bis*(β -hydroxyethyl)-sulfonium)ethyl sulfide picrylsulfonate, *bis*(β -pyridiniummethyl) sulfide, *bis*(β -pyridiniummethyl) sulfone, and vinyl(β -(*bis*(β -chloroethyl) amino)ethyl) sulfone.

The conversion of β -chloroethyl β -(*bis*(β -hydroxyethyl)sulfonium)-ethyl sulfide picrylsulfonate to the chloride was accomplished by a method suggested by Dr. W. H. Stein. The picrylsulfonate was suspended in a small quantity of acetone and water was added dropwise with agitation until the salt dissolved. The solution was chilled and an equivalent quantity of a cold solution of the dichlorocyclic dimer of *bis*(β -chloroethyl)methylamine was added. The insoluble dipicrylsulfonate of the dichlorocyclic dimer began precipitating immediately, and after standing 15 minutes, the precipitate was separated by filtration through a cotton plug. The precipitate was washed with cold water and the filtrate and washings were made up to a known volume in a volumetric flask.

¹ The work reported in this paper has been done under a contract, recommended by the National Defense Research Committee, between the Office of Scientific Research and Development and New York University.

For injection water soluble compounds were dissolved in 0.9 per cent saline, while lipid soluble compounds were generally dissolved in propylene glycol (PG). In some cases, as noted in Tables 1 and 2, thiodiglycol was employed as a solvent, but this was rarely used because of its reactivity with reactive β -halogen compounds (5). The concentration of the saline solutions was such that the injection of 0.1 cc. of the solution provided the calculated dose for a 20 gm. mouse or a 200 gm. rat. When dogs and rabbits were used the concentration of the solutions was so adjusted that 1 cc. contained the dose per kilogram of body weight. When other solvents were employed, the volume of solution injected is noted in Table 1. Similar volumes were injected of the solutions of compounds listed in Table 2.

Intravenous injections were made in a tail vein in mice, the femoral vein of rats, a marginal ear vein of rabbits and the jugular vein in dogs. Subcutaneous injections were made in the flank of mice and rats except where noted in Table 1. In applying the compounds cutaneously the compound was measured by a "drod", a $\frac{1}{4}$ cc. hypodermic syringe fitted to a micrometer screw which permitted the expression of small quantities of compound.² The drod was calibrated for each compound. The agents were applied to the clipped skin of the back while the animals were fastened to an animal board. When it was permissible, decontamination was accomplished after two hours by scrubbing the contaminated area with three cotton swabs wet with ether. When decontamination was not permissible, a collar was fastened about the neck of mice and rats to prevent self-licking, and animals were kept singly in cages.

In determining the LD_{50} , doses were decreased from a level above LD_{100} by multiples of 0.66 to a dose representing LD_0 , or conversely were increased from LD_0 by multiples of 1.5 to a dose above LD_{100} . In the mice series, 10 animals were injected at each dose, in the rat series 6 animals, and in the rabbit series 4 animals. All animals were observed for a period of 15 days and allowed food and water *ad libitum*. In calculating the LD_{50} the dose was plotted against per cent mortality on log-probability paper and a straight line was drawn by inspection to fit these points. The LD_{50} was taken as the dose at which this line crossed the 50 per cent mortality ordinate.

Among animals receiving doses in the range of the LD_{50} (i.e., from 0.5 to 2 LD_{50}), certain clinical disturbances and pathologic changes are characteristic of the systemic intoxication induced by all the sulfur and nitrogen mustards, as reported elsewhere (2). The majority of deaths occurs on the 3rd, 4th or 5th day, a type of death which has been called 'delayed'. Above the range of the LD_{50} death may be relatively rapid or may be delayed, depending on the compound and the dose. Rapid death is of course the result of an acute pharmacologic disturbance, which in some cases was the only action regardless of the dose.

RESULTS

Bis(β -chloroethyl) sulfide. The LD_{50} of *bis*(β -chloroethyl) sulfide (hereafter designated as H) for various species and by various routes are shown in Table 1. Among the species studied rats appear to be the most sensitive. Particular interest attaches to the fact that when applied cutaneously, and under conditions which preclude ingestion, the LD_{50} is relatively high in rabbits and mice (data on dogs are not available). The value given for the rabbit is only an approximation, and in some cases a dose of 100 mg./kg. was not fatal. Other workers have reported much lower LD_{50} values than the one here reported (6), but ingestion by licking may have contributed to the mortality. Pathologic examination of all fatalities in our animals has failed to show any lesions in the oral cavity or in the upper esophagus, although in animals known to have circumvented their restraints, a single lick at contaminated areas has produced death and at autopsy lesions were found in the upper gastrointestinal tract.

² This instrument was devised by Dr. P. D. McMaster.

The variations in the LD₅₀ dose in relation to solvent in the intravenous series of rabbits is possibly significant in interpreting the mechanism of systemic injury. Notice is particularly called to the difference between a solution of H in PG and

TABLE 1

The Parenteral Toxicity of H for Various Species (LD₅₀ in mg./kg.)

ANIMAL	ROUTE							
	Intravenous			Subcutaneous			Cutaneous	
	LD ₅₀	Solvent	Remarks	LD ₅₀	Solvent	Remarks	LD ₅₀	Remarks
Mouse	8.6	PG	0.05 cc. solution per 20 gm. mouse	26	PG	0.1 cc. per 20 gm. mouse	92	
Rat				3.2	PG	1 cc./200 gm. injected in flank	ca. 18	
				9.0	PG	0.5 cc./200 gm. injected in flank		
	0.7	PG	0.1 cc. per 200 gm.	7.4	sesame oil	1 cc./200 gm. injected in flank		
	3.3	neat		5.0	sesame oil	1 cc./200 gm. injected in back		
				8.5	95% alcohol	1 cc./200 gm. injected in back		
				5.2	neat	injected in flank		
Rabbit	2.7	PG	0.5 cc./kg.				ca. 100	ingestion prevented
	ca. 1.1	TG	0.5 cc./kg.					
	3.6	neat	rapid injection					
	4.5	neat	slow injection					

Abbreviations

PG: propylene glycol

TG: thiodiglycol

neat: undiluted

neat II. In the former case, H causes only diffuse pulmonary congestion, whereas when administered neat it causes severe necrotizing pulmonary lesions (2). In the transport of a substance introduced into the systemic venous circulation, the first capillary bed to be reached is in the lungs. The severe necrotiz-

ing pulmonary lesions observed after intravenous administration of neat H indicates that the venous blood contains particulate H which lodges in the pulmonary capillary bed. As a consequence of this immobilization, neighboring tissues are maximally damaged, and as long as the residue of the agent remains localized the H will be slowly hydrolyzed into the innocuous thiodiglycol. Consequently a significant fraction of the injected material never reaches sensitive tissues and the essential injuries causing systemic intoxication and delayed death are to some degree avoided. Hence the toxicity is reduced.

Compounds Related to H. It is known that the hydrolysis of H in water proceeds by a series of stepwise reactions, the first consisting of an intramolecular reaction to form the cyclical sulfonium salt, β -chloroethyl-ethylenesulfonium chloride. The second step is the reaction of the sulfonium salt with water to form β -chloroethyl β -hydroxyethyl sulfide (semi-H) and hydrochloric acid. The second reaction is considerably faster than the first and therefore it has proved impossible to isolate the cyclical sulfonium salt (7). Subsequent hydrolysis, presumably through the intermediation of a second cyclical sulfonium salt (β -hydroxyethyl-ethylenesulfonium chloride), yields thiodiglycol.

After introduction into the body H might undergo hydrolysis to form either semi-H or thiodiglycol, the carbon chain or sulfur might be oxidized, or the first or second sulfonium ion might react by conjugation with various tissue constituents. Thus toxicity could be attributed to H or to any one of these derivatives. The studies on the compounds in Table 2 were concerned with the toxicity and action of some intermediates which might be involved in the above mechanisms.

The data in Table 2 show that semi-H and thiodiglycol are relatively non-toxic and can be eliminated from participation in the toxic reactions of H.

Bimolecular and trimolecular sulfonium salts have been isolated from hydrolyses of H (4, 8). These consist of β -chloroethyl β -(bis(β -hydroxyethyl) sulfonium)ethyl sulfide chloride (H-1TG), β -hydroxyethyl β -(bis(β -hydroxyethyl) sulfonium ethyl sulfide chloride (CH-1TG) and bis- β -(bis(β -hydroxyethyl) sulfonium ethyl) sulfide dichloride (H-2TG). Of these compounds the latter two possess relatively low toxicity and thus probably do not contribute much to the toxicity of H. The remaining compound, H-1TG, had received considerable attention because of its high toxicity. However, in spite of this high toxicity, it is doubtful if it participates in the toxicity of H since its formation requires the presence of thiodiglycol, and except when employed as a solvent, thiodiglycol can arise only by hydrolysis of H. Fifteen minutes³ after the intravenous injection of an LD₅₀ dose dissolved in PG, if 50 per cent of the H remained as such and were distributed in the extracellular fluid (assuming the extracellular fluid to be 25 per cent of the body weight) the concentration of H would be 9×10^{-3} mM per liter in the rat and 34×10^{-3} mM in the rabbit. Assuming that the 50 per cent of H which had disappeared had been converted entirely to thiodiglycol in the extracellular fluid, the concentration of thiodiglycol in the rabbit would also be 34×10^{-3} mM per liter. The possibility of the formation of a bimolecular sulfonium salt at this concentration is remote. Moreover, H disappears rapidly from the blood in

³ The half-life time of H in blood at 37°C. is 14 minutes (9).

TABLE 2
Toxicity of Compounds Related to H. (LD₅₀ in mg./kg.)

COMPOUND	LD ₅₀	SOLVENT	REMARKS	
A. Compounds arising from the hydrolysis of H				
1. β -chloroethyl- β -hydroxyethyl sulfide	600 ca. 35	neat PG	Mouse Mouse	cutaneous intravenous
2. Thiodiglycol*	4-5 2 4 4 3	neat neat neat neat neat	Mouse Mouse Rat Rat Rabbit	subcutaneous intravenous intravenous subcutaneous intravenous
3. β -chloroethyl β -(bis(β -hydroxyethyl)sulfonium)ethyl sulfide picrylsulfonate	0.52 1.2 2.4	saline saline saline	Rat Mouse Mouse	intravenous intravenous subcutaneous
4. β -chloroethyl β -(bis(β -hydroxyethyl)sulfonium)ethyl sulfide chloride	ca. 15 ca. 4.5 >30 >30 6 10-15	saline saline saline saline saline saline	Mouse Rabbit Rabbit Dog Dog Rat	cutaneous intravenous cutaneous cutaneous intravenous cutaneous
5. bis(bis(β -hydroxyethyl)sulfoniumethyl) sulfide dipicrylsulfonate	50-100	saline	Mouse	subcutaneous
6. β -hydroxyethyl β -(bis(β -hydroxyethyl)sulfonium)ethyl sulfide picrylsulfonate	67 ca. 86 ca. 36	saline saline saline	Mouse Mouse Rat	intravenous subcutaneous intravenous
B. Compounds arising from the oxidation of H				
1. bis(β -chloroethyl)sulfone	ca. 50 ca. 35 >72 ca. 50	PG PG PG PG	Mouse Mouse Rat Rat	intravenous subcutaneous intravenous subcutaneous
2. Divinyl sulfone	ca. 11 ca. 16 ca. 12 ca. 14	PG PG PG PG	Mouse Mouse Rat Rat	intravenous subcutaneous intravenous subcutaneous
C. Replacement or addition derivatives of above compounds				
1. bis(β -pyridiniumethyl)sulfide	25	saline	Mouse	intraperitoneal
2. bis(β -pyridiniumethyl)sulfone	197	saline	Mouse	intraperitoneal
3. Vinyl(β -bis(β -chloroethyl)amino)ethyl sulfone†	9.0 2.55	saline saline	Mouse Rabbit	subcutaneous intravenous

* LD₅₀ for thiodiglycol is expressed in cc./kg.

† Classification of this compound among derivatives of H is arbitrary since it may also be classified as a derivative of the nitrogen mustards.

consequence of removal by blood cells and tissues, and not all the H hydrolysed appears as thiodiglycol, some being present as β -chloroethyl β -hydroxyethyl sulfide.

That H is rapidly absorbed by many tissues is certain, and such absorption, by increasing the concentration of H within the cells, would enhance the probability of bimolecular sulfonium salt formation. But since bimolecular sulfonium salt formation probably involves the cyclical sulfonium salt, which reacts rapidly with many tissues constituents, such thiodiglycol as might be formed in the cell would probably be a weak competitor.

Localization of concentrated H, for example in skin application and subcutaneous administration, offers the best conditions for bimolecular sulfonium salt formation because the extracellular fluid in the area of application or deposition may be nearly saturated with H. Even here, however, the extent of sulfonium formation appears to be slight, as judged by the fact that radioactive H-1TG comprised no more than 2.5 per cent of the extractables in pig skin after application of radioactive H (H*) (10). Hence H-1TG in the blood may be dismissed as an active agent in systemic intoxication.

Nevertheless, the high toxicity of H-1TG, particularly on the skin of rats and mice (Table 2), is a noteworthy observation. Although the compound dissociates in aqueous solution to form H and TG, apparently H-1TG is toxic *per se* since if dismutation were involved in intoxication, the LD₅₀ would be higher than that for H. The high skin toxicity indicates that a large fraction of the salt is rapidly absorbed through the skin, an unusual circumstance for a water soluble compound. Assuming that the subcutaneous LD₅₀ is a measure of the amount of agent which, after skin contamination, is absorbed through the skin, the fraction absorbed is given by the ratio of the subcutaneous LD₅₀ to the cutaneous LD₅₀, or 0.2.

In mice and rabbits intoxicated cutaneously and subcutaneously with LD₅₀ and 0.5 LD₅₀ doses of the chloride, the chief pathologic changes were: moderate to severe enteritis; mild necrosis of the liver at LD₅₀ doses; injury to the lymphoid organs, especially the spleen; and mild to moderate adrenal congestion. There was no bone marrow injury in either species. Two notable pathologic differences were observed in these species. In mice the adrenals were markedly congested while in rabbits these organs were only mildly congested. In mice the spleen was greatly enlarged (approximately 3 times the normal size) and showed marked hypertrophy of the white pulp, while in rabbits the spleen was markedly atrophic ($\frac{1}{2}$ to $\frac{2}{3}$ normal size) and the white pulp was severely involuted.

In rats intoxicated intravenously with supra LD₅₀ doses (1.4, 2.2 and $3 \times$ LD₅₀) of the picrylsulfonate, and in rabbits intoxicated intravenously with LD₅₀ doses of the chloride, terminal white blood cell counts showed the absence of significant leucopenia. In dogs the intravenous administration of 4.7 mg./kg. of the chloride and 9.0 mg./kg. of the picrylsulfonate produced a slight leucopenia in $\frac{1}{2}$ animals. Similarly, a course of 10 daily injections of 0.1 LD₅₀ dose of either the chloride or the picrylsulfonate gave rise to slight leucopenia in $\frac{1}{2}$ animals during the course of injection with counts recovering by the end of the 10-day injection

period. Animals showing slight leucopenia frequently manifested other clinical symptoms such as diarrhea and vomiting. It is conceivable that the clinical signs of intoxication are attributable to mustard which arises from dissociation of the sulfonium salt.

Oxidative processes might occur on the carbon chain or the sulfur, or both. Following the cutaneous application (11) or intravenous injection of H^* (12), a high fraction of the applied S^{35} is excreted in the urine. The excreted product occurs in the neutral sulfur fraction of the urine and has not been identified. It would appear that H is not metabolized to sulfate. Therefore, the excreted sulfur may represent any number of sulfur compounds in almost any state of oxidation. The studies of some compounds in Table 2 were made to determine the toxicity of some oxidized derivatives of H.

The toxicity of divinyl sulfone and bis(β -chloroethyl) sulfone (mustard sulfone) is capricious, the mortality data showing considerable spread. However, it seems certain that divinyl sulfone is more toxic than mustard sulfone. Peculiarly, the latter compound is more toxic in rats and mice when administered subcutaneously than when administered intravenously. This circumstance suggests that after subcutaneous injection, mustard sulfone undergoes conversion in the body fluids into the more toxic divinyl sulfone, a reaction which has been demonstrated to occur *in vitro* (13).

Toxic doses of mustard sulfone and divinyl sulfone given intravenously in rats cause death within 24 hours accompanied by pulmonary injury. But when the compounds are given subcutaneously to rats at or above LD_{50} dose, significant pathologic changes are not discoverable and neither compound produces the delayed systemic pathologic changes characteristic of H intoxication.

A few rabbit studies indicate that at doses slightly above the LD_{50} divinyl sulfone has a marked, immediate parasympathomimetic action. One rabbit receiving 20 mg./kg. of neat divinyl sulfone intravenously developed pupillary constriction and slight salivation immediately following the injection. Progressive weakness and generalized tremors followed, and after 20 minutes, parasympathetic activity persisting, the animal began to have intermittent convulsions which persisted until death at 45 minutes. A second rabbit receiving 10 mg./kg. of neat divinyl sulfone had similar immediate symptoms, parasympathomimetic action being manifest for 2 hours, and died 8 hours after injection with severe terminal convulsions. A third rabbit given 7 mg./kg. of neat divinyl sulfone failed to develop any symptoms. Convulsions also have been observed in rats receiving intravenous doses of either divinyl sulfone or mustard sulfone.

With respect to these data, it is noteworthy that mustard sulfoxide, presumably an intermediate in the chemical conversion of mustard to its sulfone, is less toxic than either H or mustard sulfone (14).

The early type of death in animals intoxicated with divinyl sulfone or mustard sulfone and the absence of pathologic lesions typical of H intoxication, are cogent arguments against the hypothesis that these oxidized derivatives play a role in the toxic action of H.

H reacts with a number of chemical groups which are found in constituents of

biological systems. It reacts with the carboxyl and amino groups of amino acids, and the nitrogen of pyridine derivatives such as nicotinic acid (5), forming derivatives which belong to the conjugative type. For the most part these derivatives are relatively non-toxic (5). However, *bis*(β -pyridiniummethyl) sulfide (Table 2) has a fairly high toxicity. This compound, along with *bis*(β -pyridiniumethyl) sulfone and vinyl β (*bis*(β -chloroethyl)amino)ethyl sulfone, were studied because of their relationship to H and divinyl sulfone.

Both *bis*(β -pyridiniummethyl) sulfone and *bis*(β -pyridiniummethyl) sulfide contain a quaternary nitrogen atom. However, they possess different chemical properties. *Bis*(β -pyridiniummethyl) sulfone exists in equilibrium with pyridine, the monopyridiniummethyl derivative and divinyl sulfone. Thus, at pH 7.5 and 25° C., *bis*(β -pyridiniummethyl) sulfone has been shown to alkylate the sulfhydryl group of cysteine and the amino group of alanine (13). *Bis*(β -pyridiniummethyl) sulfide is unable to alkylate in this manner and is apparently a stable compound. This difference in chemical properties is reflected in a difference of pharmacologic properties. The sulfone derivative has an LD₅₀ of 197 mg./kg. for mice when injected intraperitoneally whereas the sulfide derivative has an LD₅₀ of 25 mg./kg. for this species by the same route. There are no delayed deaths among mice given the sulfide, all mice which live through the first 30 minutes after injection survive the full observation period. Convulsive seizures occurred within 10 minutes after injection and deaths were apparently preceded by respiratory paralysis. No convulsions were observed after administration of the sulfone. Death time following administration of the latter compound is very similar to that after administration of divinyl sulfone, some deaths being recorded late in the first 24 hours but none during the early period.

The pharmacologic properties of *bis*(β -pyridiniummethyl) sulfide are apparently attributable to the stable character of the quaternary nitrogen atom. On the other hand, the pharmacologic properties of *bis*(β -pyridinium)ethyl sulfone are attributable to its dissociation and reactivity as an alkylating agent.

The remaining compound, vinyl β (*bis*(β -chloroethyl)amino)ethyl sulfone, is related not only to divinyl sulfone but also to the nitrogen mustards, since it contains two β -chloroethyl groups attached to a tertiary nitrogen atom. Judging from its pharmacologic action in the rabbit, this compound resembles the nitrogen mustards more than it resembles divinyl sulfone. However, in the mouse it acts like divinyl sulfone and it is included in Table 2 to complete the study of the derivatives of H. In the mouse, death is delayed whether the compound is given subcutaneously or intravenously, the majority of deaths occurring between 72 and 168 hours, with none earlier than 72 hours. In mice sacrificed at 24 hour intervals ranging from 24 to 120 hours after receiving 9.0 mg./kg. of this compound, the essential pathologic changes are mild congestion and edema of the lungs, mild enteritis appearing by 48 hours, hypertrophy of the white pulp of the spleen, increased numbers of lymphocytes with almost complete obliteration of the medulla of the thymus and mild congestion of the adrenals. It is questionable whether any of these changes, or combinations of them, are sufficient to account for death. With respect to pathologic lesions in mice the action of vinyl

β (bis(β -chloroethyl)amino)ethyl sulfone resembles that of divinyl sulfone. An account of the acute pharmacologic action of this compound in rabbits will be given elsewhere (16).

The pathological effects of the more important of the compounds reported here is reported elsewhere (1).

SUMMARY

The parenteral toxicity of bis(β -chloroethyl) sulfide (mustard gas) and certain mustard derivatives has been studied in various species. Most derivatives of mustard are relatively non-toxic. Those which are toxic are shown to act in a different manner from mustard. These data support the hypothesis that mustard acts either *per se* or in the form of its first aqueous reaction product, β -chloroethyl-ethylene sulfonium chloride.

We are indebted to Elsa Addis (Karnofsky), Mildred Bevelander, Betty Lou Ellis, Vera Hoie, Vera James, Penelope Smith and Lee Summers in the conduct of the experiments.

BIBLIOGRAPHY

1. SMITH, H. W.: OSRD Formal Report 1717.
2. GRAEF, I., KARNOFSKY, D. A., JAGER, B. V., KRICHESKY, B. AND SMITH, H. W.: Amer. J. Path. 24, 1, 1948.
3. GRANT, W. M., AND KINSEY, V. E.: J. Amer. Chem. Soc. 68:2075, 1946.
4. STEIN, W. H., MOORE, S., AND BERGMANN, M.: J. Organic Chem. 11: 664, 1946.
5. BERGMANN, M., ET AL.: Personal communication.
6. CAMERON, G. R.: Personal communication.
7. PRICE, C. C., AND WAKEFIELD, L. B.: J. Organic Chem. 12: 232, 1947.
8. RYDON, H. N.: Personal communication.
9. GRANT, W. M., AND KINSEY, V. E.: J. Biol. Chem. 165: 495, 1946.
10. HENRIQUES, F. C., JR., MORITZ A. R., AND PATTERSON, L. A.: Personal communication.
11. YOUNG, L.: Personal communication.
12. BOURSNEILL, J. C., COHEN, J. A., DIXON, M., FRANCIS, G. E., GREVILLE, G. D., NEEDHAM, D. M., AND WORMALL, A.: Biochem. J. 40: 756, 1946.
13. STAHLMAN, M. A., GOLUMBIC, C., STEIN, W. H., AND FRUTON, J. S.: J. Organic Chem. 11: 719, 1946.
14. University of Chicago Toxicity Laboratory. Personal communication.
15. MOORE, S., STEIN, W. H., AND FRUTON, J. S.: J. Organic Chem. 11: 675, 1946.
16. ANSLOW, W. P., JR., KARNOFSKY, D. A., JAGER, B. V., AND SMITH, H. W.: THIS JOURNAL 91: 224, 1947.

THE EFFECTS OF INTRAVENOUS INJECTION OF TETRA-ETHYL-AMMONIUM CHLORIDE ON THE INTRA-ARTERIAL BLOOD PRESSURE AND OTHER PHYSIOLOGIC VARIABLES IN MAN

GEORGE E. BROWN, JR., EARL H. WOOD, AND EDWARD H. LAMBERT

WITH THE TECHNICAL ASSISTANCE OF ROY L. ENGSTROM

Department of Medicine, Mayo Clinic, Section on Physiology, Mayo Foundation and Mayo Clinic, Rochester, Minnesota

Received for publication November 10, 1947

It has been demonstrated by Acheson and Moe (1) that the tetra-ethyl-ammonium ion blocks the transmission of nerve impulses through autonomic ganglia. The autonomic blockade produced by the injection of suitable doses of this quaternary ammonium compound in man results in a decrease of arterial blood pressure, an increase of blood flow through the extremities, an increase of heart rate, a decrease of gastro-intestinal motility and postural hypotension in addition to other effects (2-4). While the general effects of this drug on man have been reported, exact details of its action and time course of its action have not been presented.

Since the action of the tetra-ethyl-ammonium ion in man has proved to be of clinical importance, further investigation of the effects of the drug has been undertaken, utilizing the methods developed during the war for the continuous recording of multiple physiologic variables in man during exposure to centrifugal force (5).

METHODS. Eight healthy men between the ages of eighteen and thirty-four years were used as subjects. No experiment was carried out less than one and a half hours after a meal and studies were conducted in a room the temperature of which was maintained between 77° and 79° F. With the subject supine continuous and simultaneous photographic recordings were made of the arterial blood pressure, venous pressure, heart rate, electrocardiogram, respiration, ear opacity pulse, blood content of the ear and intrarectal pressure. In a few subjects plethysmographic measurement of changes in leg volume were recorded.

Pressures were recorded by means of resistance wire, strain gauge manometers as described by Lambert and Wood (6). Arterial pressure was measured from the right radial artery at the wrist. The site of puncture and the manometer (Model P6-15D-250¹) were adjusted to the level of the third intercostal space at the sternum. The natural frequencies of the manometer systems used for recording arterial pressure ranged from 65 to 99 cycles per second.

Venous pressure in the greater saphenous vein at the ankle was recorded using a similar strain gauge manometer (Model P6-4D-250¹). Intrarectal pressure was measured by means of an air-filled balloon and catheter inserted 12 cm. above the anal ring. The catheter was connected to a strain gauge manometer of suitable sensitivity (Model P6-4D-250).¹

The electrocardiogram was recorded from chest leads located just below each nipple. The electrocardiograph activated an instantaneous recording cardiometer (7) so that

¹ Statham Laboratories, Los Angeles, California.

a continuous record of the heart rate was obtained. Respiration was recorded by means of a basal metabolism mouthpiece containing a thermocouple (8). The ear opacity pulse and the blood content of the ear were measured by means of barrier layer photoelectric ear plethysmographs (8). Changes in leg volume were recorded by means of a leg plethysmograph (9).²

Records were made during control periods ranging from twenty-two to fifty-four minutes before the drug was given. Tetra-ethyl-ammonium chloride (etamon chloride) was then injected into the left antecubital vein. Subjects 1, 2 and 3 were given the drug in a single dose of from 5.5 to 7.7 mg. per kilogram of body weight injected slowly over a period of from one and a half to three and a half minutes. Subjects 4 through 8 were given the drug in five equally divided doses. Each dose was injected rapidly-over a period of less than three seconds. One or two minutes were allowed to elapse between the first and second dose and one minute between subsequent doses. The total dose ranged between 5.7 and 7.7 mg. per kilogram of body weight. Recordings of the various physiologic variables were taken continuously from these eight subjects for periods of twenty-five to seventy-two minutes after the start of the injection of the drug.

RESULTS. The subjective symptoms experienced after intravenous injection of tetra-ethyl-ammonium chloride were similar to those described by Lyons and his co-workers (2). A metallic taste occurring at an average time of nineteen seconds after the start of the injection was followed by tingling and coldness in the extremities eight seconds later. Dryness of the mouth was noted by some while loss of visual accommodation and a generalized feeling of lassitude were experienced by all subjects. When the drug was given in five equally divided doses, each injection was followed by the same train of events as followed a single dose, though the coldness and tingling were usually less distinct possibly because of persistence from the previous injections.

The systolic blood pressure decreased in all subjects, the decrease beginning at an average time of forty-six seconds (range twenty to eighty-five seconds) after the start of the injection. A transient increase of pressure occurred in two subjects immediately after the injection of the drug. If these subjects are excluded, the average time until the initial decrease in pressure occurred was thirty-seven seconds. Figure 1 illustrates the changes in average systolic pressure over fifteen second intervals at various periods after the injection of the drug. The average decrease of pressure below the control values was found to be statistically significant (p less than 0.02) for all periods up to twenty-five minutes after injection of the drug. A slight average decrease in systolic pressure persisted for the duration of the approximately forty minute period in which observations were continued.

The spontaneous variations in blood pressure were somewhat reduced after injection of the drug. The average standard deviation of the systolic pressure of successive pulse waves over fifteen second intervals during the control periods was 2.8 mm. of mercury. One minute after injection of the drug was completed the beat-to-beat variations in blood pressure were reduced so that the standard deviation was decreased to 1.3 mm. of mercury (table I).

² Plethysmographic studies were made possible through the assistance of O. L. Slaughter, M.D.

Table I also shows the effect on the systolic pressure of the first two of five equally divided doses of the drug given over a five minute period. On the aver

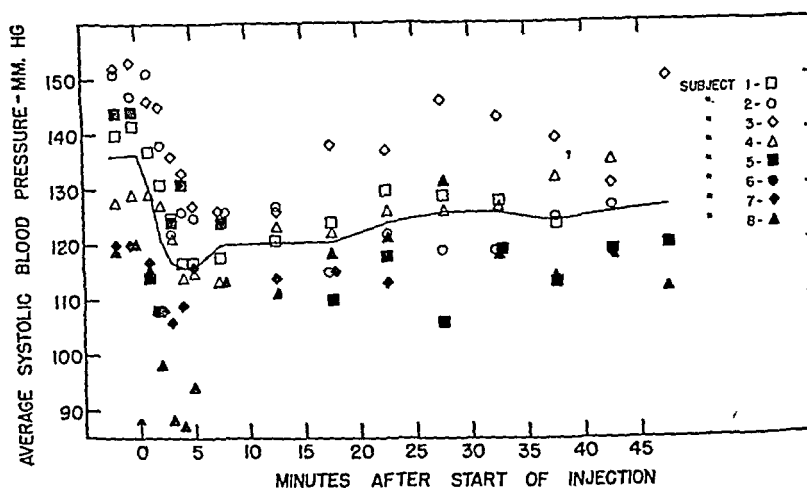


Fig. 1

The effect of intravenous injection of tetra-ethyl-ammonium chloride on systolic blood pressure in eight normal men. Blood pressure recorded directly from the radial artery the wrist.

TABLE I

The effect of tetra-ethyl-ammonium chloride* on the spontaneous variations in systolic blood pressure measured in millimeters of mercury

SUBJECT	CONTROL PERIOD		1 MIN. AFTER 1/2 MAX. DOSE		1 MIN. AFTER COMPLETION OF INJECTION		15 MIN. AFTER COMPLETION OF INJECTION	
	Average pressure	Standard deviation†	Average pressure	Standard deviation†	Average pressure	Standard deviation†	Average pressure	Standard deviation†
4	129	1.9	123	2.9	113	1.7	112	2.0
5	144	4.8	124	1.3	124	1.5	118	1.9
7	120	2.9	109	0.3	106	1.7	113	1.4
8	120	1.5	99	1.5	88	0.2	121	1.4
Average values.....	128	2.8	114	1.5	108	1.3	116	1.7

* The drug was injected intravenously in five equally divided doses spaced over a period of five minutes. The total dose was 5.7 to 7.7 mg. per kilogram of body weight.

† Standard deviation of the systolic pressure of successive pulse waves from the average systolic pressure during a fifteen second period.

age the first two doses of the drug (40 per cent of the total dose) produced decrease in systolic pressure amounting to 70 per cent of that produced by total of five doses.

The effect of the drug on diastolic pressure was variable. A decrease in diastolic pressure of 4 to 14 mm. of mercury occurred in four of the seven subjects within the first five minutes after the start of the injection while in three subjects increases of 4 to 12 mm. of mercury occurred. No relationship was demonstrated between the effect on diastolic pressure and the size of the dose. The difference between the control values for diastolic pressure and the values during various periods after injection of the drug were statistically not significant (p values exceeded 0.13 in all instances).

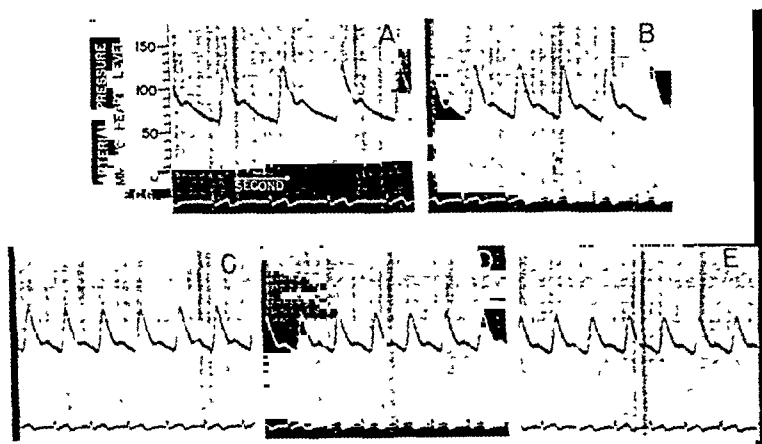


FIG. 2

The effect of four successive intravenous injections of 90 mg. of tetra-ethyl-ammonium chloride on intra-arterial pressure pulse contours recorded from the radial artery in a normal man (Body Weight: 58 Kg.). A. One minute before start of injection. B. One minute after first 90 mg. C. One minute after second 90 mg. D. One minute after third 90 mg. E. One minute after fourth 90 mg. Successive doses were separated by one minute intervals.

The changes in pulse contour after injection of the drug are illustrated in figure 2. The changes in mean blood pressure determined by planimetric measurement of the pulse waves ranged from a decrease of 20 mm. of mercury to an increase in one instance of 3 mm. of mercury. The average decrease of mean pressure during the period from three to five minutes after the start of the injection was 8 ± 2.9^3 mm. of mercury. The pulse pressure was decreased in all subjects. The average decrease in pulse pressure during the period from three to five minutes after injection of the drug was 24 ± 4.1^3 mm. of mercury.

The heart rate was increased in all subjects after the injection of tetra-ethyl-ammonium chloride. This increase in rate began on the average at thirty-seven seconds (range twenty to sixty seconds) after the start of the injection. The changes in heart rate for the eight subjects are shown in figure 3. The differences between the average heart rate during fifteen second intervals in the control

³ The figure following the \pm sign is the standard error of the mean, $n = 7$.

period and during similar periods after the injection of the drug were statistically significant (p value less than 0.009) for all periods from two to twenty minutes. A slight increase in average heart rate persisted for the duration of the approximately forty minute period in which observations were continued after injection of the drug.

The heart rate usually responded in stepwise fashion to divided doses of the tetra-ethyl-ammonium salt. However, in subjects 7 and 8 the effect of the drug was apparently maximal after the fourth dose since no further increase occurred

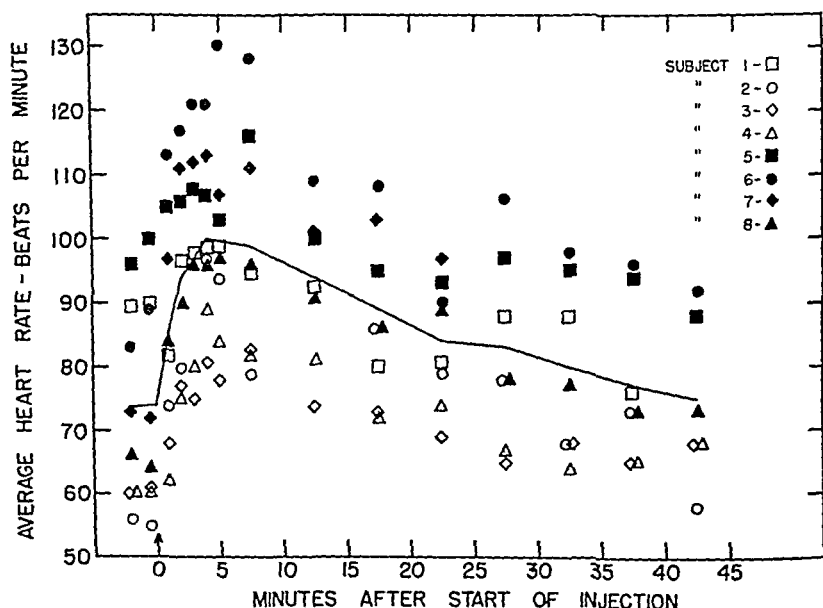


FIG. 3

The effect of intravenous injection of tetra-ethyl-ammonium chloride on the heart rate in eight normal men.

subsequent to the fifth injection. The spontaneous variations of heart rate from beat to beat were markedly diminished after the injection of tetra-ethyl-ammonium chloride. The average standard deviation for successive heart beats over fifteen second periods before injection of the drug was 3.3 beats per minute. One minute after injection of the drug was completed the beat-to-beat variations in the heart rate were reduced to such an extent that the average standard deviation was 0.2 beats per minute (table II).

Table II also shows the effect on the heart rate of the first two of five equally divided doses of the drug given over a five minute period. On the average, the first two doses of the drug (40 per cent of the total dose) produced an increase in heart rate amounting to 81 per cent of that produced by the total dose.

The drug produced a slight increase in venous pressure, measured in the greater saphenous vein at the ankle, in each of four subjects. This increase began on the average at thirty seconds (range twenty-five to forty seconds) after the start of the injection of tetra-ethyl-ammonium chloride. The average maximal increase of 2.0 mm. of mercury (range 1 to 3 mm. of mercury) occurred at three minutes after the start of the injection. Venous pressure remained slightly elevated above control values in all subjects for forty-five minutes after the drug was given. After the administration of tetra-ethyl-ammonium chloride, pulsations synchronous with the heart beat became discernible in the venous pressure in each of the four subjects.

TABLE II

The effect of tetra-ethyl-ammonium chloride on the spontaneous variations in heart rate (beats per minute)*

SUBJECT	CONTROL PERIOD		1 MIN. AFTER ‡ MAX. DOSE		1 MIN. AFTER COMPLETION OF INJECTION		15 MIN. AFTER COMPLETION OF INJECTION	
	Average rate	Standard deviation†	Average rate	Standard deviation†	Average rate	Standard deviation†	Average rate	Standard deviation†
4	60	4.5	75	1.1	84	0.1	74	1.6
5	100	3.3	108	0.2	116	0.2	93	1.7
6	88	3.2	128	0.3	130	0.2	90	0.3
7	72	3.1	108	1.1	113	0.2	97	1.3
8	64	2.2	90	0.5	97	0.5	89	1.0
Average values.....	77	3.3	102	0.6	108	0.2	89	1.2

* The drug was injected intravenously in five equally divided doses spaced over a period of five minutes. The total dose was 5.7 to 7.7 mg. per kilogram of body weight.

† Standard deviation of the rate of a single heart beat from the average rate of the beats occurring during a fifteen second period.

Changes in the volume of the leg were recorded in four subjects. An increase in volume beginning at an average time of twenty-eight seconds (range twenty-five to thirty seconds) after the start of the injection of the drug occurred in all instances. The average increase in leg volume one minute after the fifth dose of the drug was 35 cc. (range 20 to 50 cc.).

Mean intrarectal pressure was not altered by the injection of tetra-ethyl-ammonium chloride. In all four subjects in whom intrarectal pressure was recorded, irregular contractions of the bowel were abolished at an average time of thirty-nine seconds (range twenty to sixty seconds) after the drug was injected. The regular waves synchronous with respiration were maintained and in addition small pulsations synchronous with the heart beat become more evident; these effects persisted for the duration of the experiment (twenty-five to seventy-two minutes) in these subjects.

The intravenous injection of tetra-ethyl-ammonium chloride was followed in every instance by an increase in the blood content of the ear. The increase in

period and during similar periods after the injection of the drug were statistically significant (p value less than 0.009) for all periods from two to twenty minutes. A slight increase in average heart rate persisted for the duration of the approximately forty minute period in which observations were continued after injection of the drug.

The heart rate usually responded in stepwise fashion to divided doses of the tetra-ethyl-ammonium salt. However, in subjects 7 and 8 the effect of the drug was apparently maximal after the fourth dose since no further increase occurred

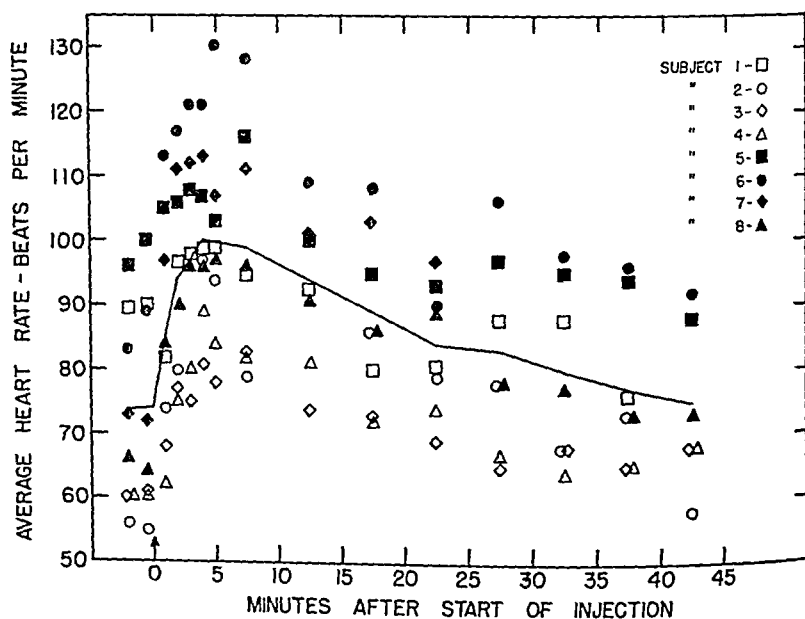


FIG. 3

The effect of intravenous injection of tetra-ethyl-ammonium chloride on the heart rate in eight normal men.

subsequent to the fifth injection. The spontaneous variations of heart rate from beat to beat were markedly diminished after the injection of tetra-ethyl-ammonium chloride. The average standard deviation for successive heart beats over fifteen second periods before injection of the drug was 3.3 beats per minute. One minute after injection of the drug was completed the beat-to-beat variations in the heart rate were reduced to such an extent that the average standard deviation was 0.2 beats per minute (table II).

Table II also shows the effect on the heart rate of the first two of five equally divided doses of the drug given over a five minute period. On the average, the first two doses of the drug (40 per cent of the total dose) produced an increase in heart rate amounting to 81 per cent of that produced by the total dose.

The drug produced a slight increase in venous pressure, measured in the greater saphenous vein at the ankle, in each of four subjects. This increase began on the average at thirty seconds (range twenty-five to forty seconds) after the start of the injection of tetra-ethyl-ammonium chloride. The average maximal increase of 2.0 mm. of mercury (range 1 to 3 mm. of mercury) occurred at three minutes after the start of the injection. Venous pressure remained slightly elevated above control values in all subjects for forty-five minutes after the drug was given. After the administration of tetra-ethyl-ammonium chloride, pulsations synchronous with the heart beat became discernible in the venous pressure in each of the four subjects.

TABLE II

The effect of tetra-ethyl-ammonium chloride on the spontaneous variations in heart rate (beats per minute)*

SUBJECT	CONTROL PERIOD		1 MIN. AFTER ½ MAX. DOSE		1 MIN. AFTER COMPLETION OF INJECTION		15 MIN. AFTER COMPLETION OF INJECTION	
	Average rate	Standard deviation†	Average rate	Standard deviation†	Average rate	Standard deviation†	Average rate	Standard deviation†
4	60	4.5	75	1.1	84	0.1	74	1.6
5	100	3.3	108	0.2	116	0.2	93	1.7
6	88	3.2	128	0.3	130	0.2	90	0.3
7	72	3.1	108	1.1	113	0.2	97	1.3
8	64	2.2	90	0.5	97	0.5	89	1.0
Average values.....	77	3.3	102	0.6	108	0.2	89	1.2

* The drug was injected intravenously in five equally divided doses spaced over a period of five minutes. The total dose was 5.7 to 7.7 mg. per kilogram of body weight.

† Standard deviation of the rate of a single heart beat from the average rate of the beats occurring during a fifteen second period.

Changes in the volume of the leg were recorded in four subjects. An increase in volume beginning at an average time of twenty-eight seconds (range twenty-five to thirty seconds) after the start of the injection of the drug occurred in all instances. The average increase in leg volume one minute after the fifth dose of the drug was 35 cc. (range 20 to 50 cc.).

Mean intrarectal pressure was not altered by the injection of tetra-ethyl-ammonium chloride. In all four subjects in whom intrarectal pressure was recorded, irregular contractions of the bowel were abolished at an average time of thirty-nine seconds (range twenty to sixty seconds) after the drug was injected. The regular waves synchronous with respiration were maintained and in addition small pulsations synchronous with the heart beat become more evident; these effects persisted for the duration of the experiment (twenty-five to seventy-two minutes) in these subjects.

The intravenous injection of tetra-ethyl-ammonium chloride was followed in every instance by an increase in the blood content of the ear. The increase in

ear volume began on the average at thirty-four seconds (range twenty-five to sixty-five seconds) after the start of the injection of the drug. The maximal increase occurred at an average time of 147 seconds (range 120 to 180 seconds) after the start of the injection in subjects who received a single dose. In subjects who received the drug in divided doses, the maximal increase in blood content of the ear occurred on the average at 205 seconds (range 120 to 240 seconds) after the beginning of the injection period, at which time an average of 60 per cent (range 40 to 80 per cent) of the total dose had been given.

COMMENT. Tetra-ethyl-ammonium chloride, as a consequence of its ability to block transmission of impulses through autonomic ganglia, produces marked alterations of the physiologic mechanisms which are under autonomic control. The application of methods of continuously recording multiple physiologic variables is of value in the study of the effects of this drug on man since it greatly facilitates the comparison of simultaneous effects of the drug from moment to moment on several physiologic mechanisms.

The release of the peripheral portions of the circulatory system from the influence of vasoconstrictor impulses can account for most of the effects on blood pressure which have been described. In the supine position maintained throughout this study systolic blood pressure was significantly decreased by injection of the drug while diastolic pressure was not significantly affected. Acheson and Moe (1) explained these changes on the basis of a decrease of peripheral resistance. Peripheral vasodilatation and increased blood flow (3) afford adequate explanation for the increase of leg and ear volume which was found in the present study. The slight increase of venous pressure in the saphenous vein at the ankle and the appearance of pulse waves in the venous pressure recordings are additional indications of the presence of a wide-open peripheral vascular bed after injection of the drug.

In confirmation of the results of others the heart rate was found to be increased in every instance after injection of the drug. This alteration of heart rate has been attributed to blockade of the inhibitory pathways, which in man predominate over the accelerator influences. The marked reduction of the normal spontaneous variations of heart rate from beat to beat after injection of tetra-ethyl-ammonium chloride is an additional consequence of this blockade of the normally present sympathetic and vagus influence on the rate of the heart. The maximal effects on the rate and spontaneous variations of the heart beat often occurred before the full dose of the drug had been administered although blood pressure was further decreased by completion of the injection. This suggests that a larger amount of the drug was required to block sympathetic vasoconstrictor impulses effectively than to block parasympathetic cardio-inhibitory impulses.

The abolition of the slow variations of intrarectal pressure is confirmatory evidence of a decrease of gastro-intestinal motility as has been reported by others (4). This effect occurred promptly and often completely with amounts of tetra-ethyl-ammonium chloride as small as 100 mg. administered intravenously. The effect of the drug on the motility of the rectum persisted much longer than the effect on the cardiovascular system. Loss of accommodation with its result-

ing visual disturbances was one of the most annoying side effects of this drug. This effect likewise persisted for a longer period than the effects on the circulatory system.

The local effects of the drug on a nerve trunk were demonstrated accidentally when a small portion of a dose was inadvertently injected into the antecubital space in one subject. A marked flexor spasm of the homolateral fingers, hand and wrist associated with fasciculation of the muscles at the base of the thumb occurred and persisted for four hours. Altered sensation in the hand persisted for eighteen hours. Similar experiences have been reported by Lyons and associates (2). No other persistent ill effects from the injection of tetra-ethyl-ammonium chloride were encountered in the present studies.

SUMMARY

By the application of methods of continuously recording several physiologic variables it has been demonstrated that the intravenous injection of 5.5 to 7.7 mg. of tetra-ethyl-ammonium chloride (etamon) per kilogram of body weight in man is followed by a decrease of the intra-arterial systolic blood pressure, an increase of the heart rate, an increase of volume of the leg and ear and a decrease of gastro-intestinal motility. These changes occurred within a period of thirty-nine seconds after the start of the injection of the drug. The effects on the motility of the rectum continued as long as seventy-two minutes after the drug was given while the effects on blood pressure and heart rate were more transient. In this series of eight subjects, the average reductions of arterial pressure and increases of heart rate over fifteen second intervals were statistically significant for a period of twenty to twenty-five minutes after injection of the drug. Slight average decreases in systolic pressure and increases in heart rate persisted for periods of longer than forty minutes after injection of the drug.

REFERENCES

1. ACHESON, G. H. AND MOE, G. K.: *THIS JOURNAL* 87: 220, 1946.
2. LYONS, R. H., MOE, G. K., NELIGH, ROSALIE B., HOOBLER, S. W., CAMPBELL, K. V., BERRY, R. L. AND RENNICK, BARBARA R.: *Am. J. M. Sc.* 213: 315, 1947.
3. MALTON, D., HOOBLER, S. W., BALLANTINE, H. T., JR., NELIGH, R. B., COHEN, SAUL AND LYONS, R. H.: *Federation Proc.* 6: 353, 1947.
4. NELIGH, R. B., HOLT, J., LYONS, R. H., MOE, G. K. AND HOOBLER, S. W.: *Proc. Am. Federation Clin. Research.* 3: 20, 1947.
5. WOOD, E. H., LAMBERT, E. H., BALDES, E. J. AND CODE, C. F.: *Federation Proc.* 5: 327, 1946.
6. LAMBERT, E. H. AND WOOD, E. H.: *Proc. Soc. Exper. Biol. & Med.* 64: 186, 1947.
7. STURM, R. E. AND WOOD, E. H.: *Federation Proc.* 5: 102, 1946; also *Rev. Scient. Instruments.* 18: 771, 1947.
8. The effects of acceleration and their amelioration. Monograph edited by Subcommittee on Acceleration, Committee on Aviation Medicine, National Research Council. Unpublished data.
9. SLAUGHTER, O. L. AND LAMBERT, E. H.: *Federation Proc.* 6: 203, 1947.

EFFECT OF PODOPHYLLIN ON TRANSPLANTED MOUSE TUMORS^{1,2}

MORRIS BELKIN²

Department of Pharmacology, Medical College of South Carolina, Charleston

Received for publication November 10, 1947.

It has been reported by Kaplan (1) and by King and Sullivan (2, 3) that the use of a suspension of podophyllin in oil is an effective method for the treatment of condylomata acuminata. Some of the primary cytological changes produced by podophyllin, as described by the latter investigators, were alteration in mitosis, principally of the metaphase; pycnosis, spongy swelling and cytoplasmic vacuolization. Since some of the cellular changes following the administration of podophyllin strongly resemble those produced by colchicine, and inasmuch as colchicine has for many years been known to affect mitosis of normal and malignant cells, it was considered of interest to test the action of podophyllin on transplanted mouse tumors.

Podophyllin (U.S.P.X.) dispersed in sesame oil was given subcutaneously in dose of 20 mgm. per kgm. to twelve Swiss albino mice carrying fifteen day old implants of sarcoma 180. Injections were made every three or four days for a period of two weeks on the side opposite the tumor. Four such injections were made in this group. A similar group of mice served as controls and were given equivalent amounts of sesame oil alone. On every third day the three diameters of the tumors were measured by means of calipers, and the mean of these diameters was used to calculate tumor volume, on the assumption that the tumors were spherical. Two measurements were taken before treatment with podophyllin was begun.

In figure 1, the rate of growth of the tumors in the two groups is shown. The controls grew typically, but the tumors in the mice receiving podophyllin exhibited a prompt decrease in growth rate. At the end of two weeks, when the experiment was terminated, the average volume of the treated tumors was approximately one seventh that of the controls.

In another experiment similarly conducted, the effect of podophyllin was tested on a mammary adenocarcinoma. This tumor arose spontaneously in a female C₃H mouse in these laboratories and had been carried in transplant for almost three years in this strain. As shown in figure 2, the terminal volumes of the 12 podophyllin treated mammary tumors was approximately two thirds that of the 12 controls. Because of the toxic effects of the podophyllin, the mice in this experiment received but 3 injections of the drug.

For both kinds of tumors used in these experiments, the most prominent and consistent histologic finding following podophyllin administration was extensive

¹ Presented at the Thirty-first Annual Meeting of the American Society for Pharmacology and Experimental Therapeutics, Chicago, May 20, 1947.

Abstract in Federation Proceedings, Vol. 6, p. 303, March 1947.

² Present address: National Cancer Institute, Bethesda, Maryland.

³ This work was supported in part by a grant from the S. C. Division of the American Cancer Society.

necrosis In figure 3 a typical case of this phenomenon is shown, as found in sarcoma 180, and, in figure 5, as found in the mammary adenocarcinoma. For comparison, microscopic sections of control tumors of sarcoma 180 and of the mammary adenocarcinoma are shown in figures 4 and 6, respectively.

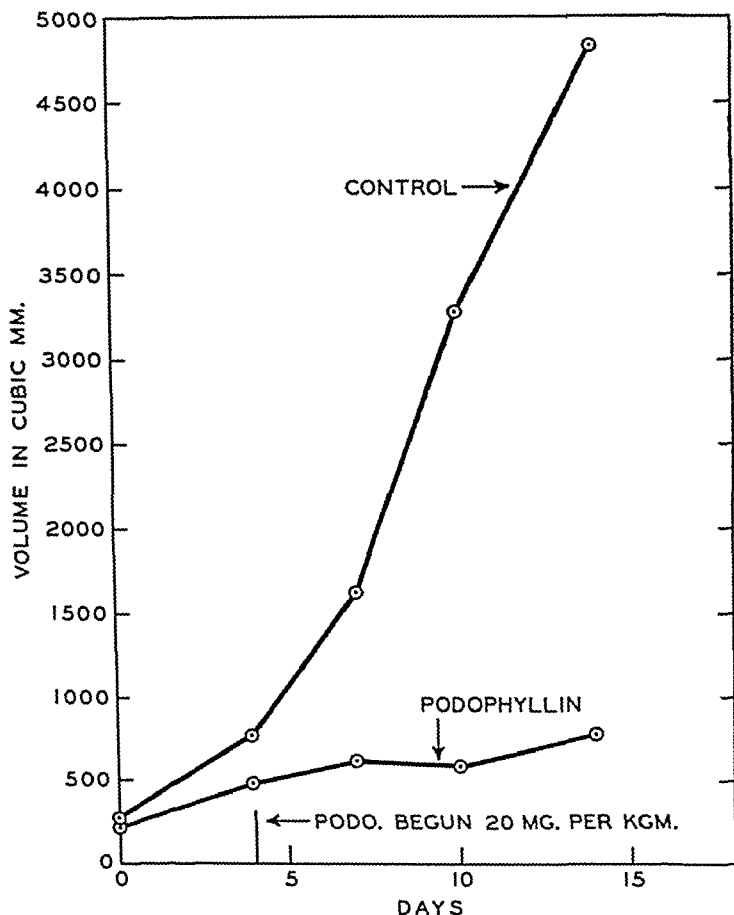


FIG 1 EFFECT OF PODOPHYLLIN ON GROWTH RATE OF SARCOMA 180 IN SWISS ALBINO MICE

Hemorrhage was not a constant feature in either type of tumor but was encountered occasionally and varied in degree from moderate to severe. To the extent to which it was observed, it was more frequent in the mammary adenocarcinoma than in the sarcoma.

Cytologically, hydropic degeneration and vacuolization of the cytoplasm were more pronounced in sarcoma 180 than in the mammary tumor. Characteristic

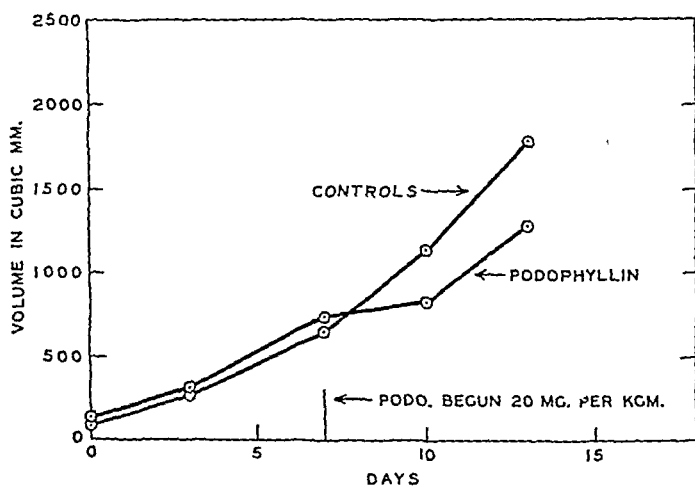


FIG. 2. EFFECT OF PODOPHYLLIN ON GROWTH RATE OF TRANSPLANTED MAMMARY ADENOCARCINOMA IN C_3H MICE

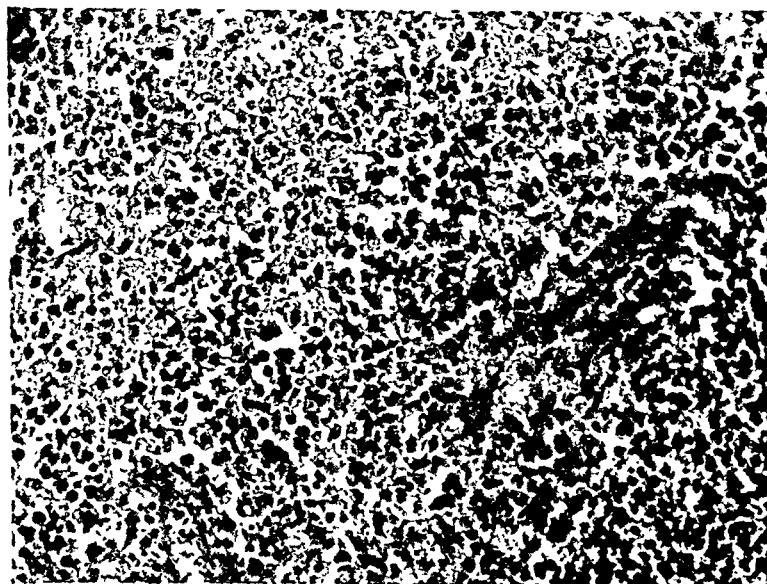


FIG. 3 EFFECT OF PODOPHYLLIN ON SARCOMA 180 MAGNIFICATION $\times 300$

nuclear alterations found in both types of tumor were diminution in number of mitoses, and abundant pycnosis and karyorrhexis. Arrest of mitosis, before or at the metaphase, was a frequently observed phenomenon in both tumors and resembled the effect of colchicine in this regard.

Administration of podophyllin produced some toxicity. In 3 to 4 hours the mice showed varying degrees of malaise, and in 8 to 10 hours the majority had diarrhea. It was difficult to decide from these observations whether the diarrhea was due entirely to systemic absorption or in part due to the fact that the mice

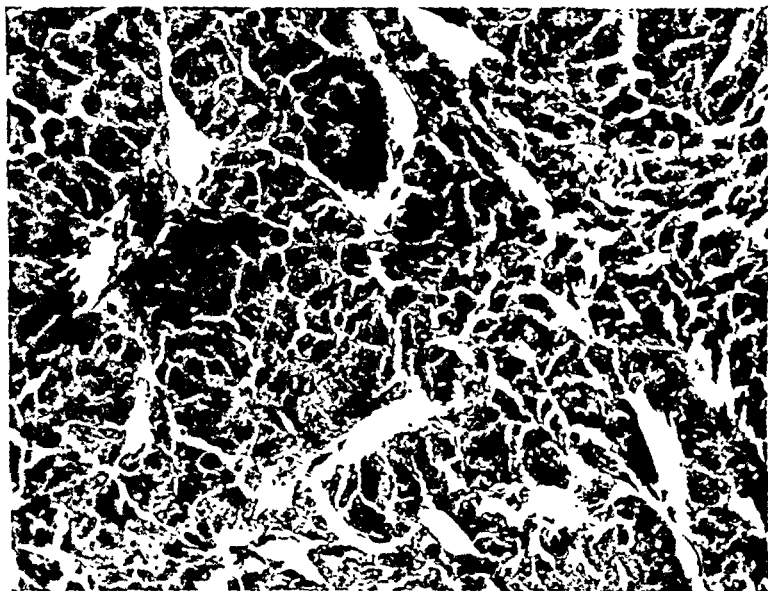


FIG. 6. TRANSPLANTED MAMMARY ADENOCARCINOMA IN C_2H STRAIN. CONTROL.
MAGNIFICATION $\times 300$

licked the oil, a small amount of which occasionally leaked out through the injection site. In this connection, however, it is of interest that in experiments by Viehover and Mack (4) in which 20 mgm. of amorphous podophyllotoxin, one of the constituents of podophyllin, was given subcutaneously to dogs, intestinal evacuation was reported after 7 hours.

For about 24 hours following administration of podophyllin, the mice remained relatively quiet, and it was observed that during this period their food consumption was below normal. Since it has been shown by Bischoff and Long (5) and by Tannenbaum (6), that food restriction by itself will, under certain conditions, cause a retardation of tumor incidence and growth, the possible effect of such restriction must be considered.

To secure some evidence bearing on this point, twelve Swiss mice carrying im-

plants of sarcoma 180 were given food (dog chow) *ad libitum* for a number of days and the amount consumed every 24 hours was measured. The chow was then restricted to 60 percent of their measured daily consumption, and the mice were maintained on this restricted diet for two weeks. Unlimited water was available at all times. The tumors were measured every three days as previously described. A reduction in terminal volume of about 30 percent resulted in these tumors when compared with the terminal volumes of the sarcoma 180 controls shown in figure 1.

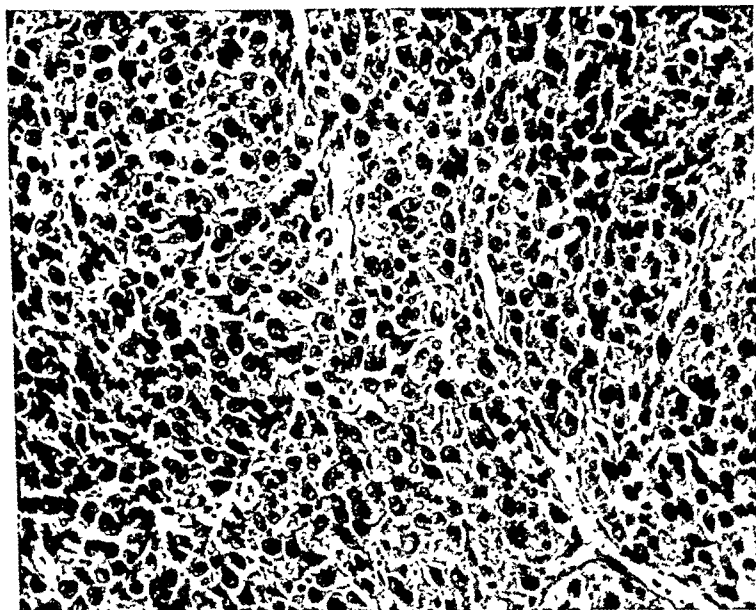


FIG. 7. SARCOMA 180 AFTER TWO WEEKS ON RESTRICTED FOOD INTAKE.
MAGNIFICATION $\times 300$

The purpose of this approach was to ascertain the effect of a restriction in food intake on the growth rate of sarcoma 180. The 40 percent reduction in food was a relatively severe one; also the restriction was maintained continuously throughout the experimental period. The podophyllin treated mice, on the other hand, exhibited a temporary, intermittent curtailment of food intake; but no data were available bearing on the possible effect of this degree and type of food restriction on the growth of tumors. However, the experience of investigators in this field affords the opinion that such occasional reduction in food consumption, as shown by the mice in these experiments, would have a negligible effect on tumors already established and actively growing (7).

This opinion is borne out by the fact that at the termination of the experiment, the microscopic appearance of the tumors growing under the above conditions of

food restriction was that of typical sarcoma 180 (figure 7) indicating that any curtailment of food intake experienced by the podophyllin treated mice did not contribute to the induced cellular and tissue changes described above. Areas of necrosis were found, but these were spontaneous in origin and are to be expected in transplanted tumors of this age (3 weeks).

It was found by King and Sullivan (1) that in normal rabbit skin the cytological effects following a single application of podophyllin were transitory, with restoration of essentially normal epidermis in 4 to 6 days. To determine whether tumor cells responded in analogous fashion to this drug, an experiment was performed in which a single injection of podophyllin in sesame oil, in a dose of 20 mgm. per kgm., was given subcutaneously to Swiss mice carrying implants of sarcoma 180. Pairs of podophyllin treated mice, with pairs of untreated controls, were then sacrificed at intervals of 12, 24, 48, 72 and 96 hours. The nuclear and cytoplasmic changes following treatment with podophyllin as described above were most apparent 12 to 24 hours after administration of the drug; with diminution of effect already evident by 48 hours. The amount of early acute necrosis present, even after 12 hours, was impressive.

In a number of the histologic sections of tumors from the experiments with repeated doses there were observed areas of old and fresh induced necrosis, indicating that the specific podophyllin effect was exerted following each administration of the drug. Also, the extent of the necrosis in animals following several injections was much greater than in animals receiving one or two injections. In the case of the former, there were some instances of confluent necrotic areas in various states of necrosis.

The reported resistance (2) to repeated application of podophyllin which appears to become established in normal rabbit skin apparently does not take place in the case of the tumors studied.

In the present experiments, administration of podophyllin had to be made at intervals of three to four days because of the toxic effects encountered when the drug was given more frequently. It may be possible that greater and more sustained tumor necrotizing effects may be produced following the use, individually or in combination, of the various components known to be present in podophyllin.

SUMMARY

1. Podophyllin dispersed in sesame oil, given subcutaneously in doses of 20 mgm. per kgm. to mice bearing implants of sarcoma 180 or a mammary adenocarcinoma, produced retardation of tumor growth. The terminal volume of the sarcoma was about one-seventh and that of the carcinoma about two-thirds of their respective controls.
2. Extensive induced necrosis was the most prominent histologic finding for both types of tumors.
3. Characteristic nuclear changes found in both kinds of tumors were diminution in mitoses, pycnosis, and karyorrhexis. Arrest of mitosis, before the metaphase, was also found in both types, and resembled colchicine effects.

4. Hydropic degeneration and vacuolization of the cytoplasm was more evident in sarcoma 180.

5. Resistance to repeated administration does not appear to develop, judged by the appearance of the tumors after several injections of the drug.

Dr. W. M. Cannon, Department of Pathology, Medical College of the State of South Carolina, assisted in the interpretation of the microscopic material and this cooperation is gratefully acknowledged.

REFERENCES

1. KAPLAN, I. W.: New Orleans Med. Surg. J. 94: 388, 1942.
2. KING, L. S., AND SULLIVAN, M.: Science 104: 244, 1946.
3. KING, L. S., AND SULLIVAN, M.: Arch. Path. 43: 374, 1947.
4. VIEHOVER, A., AND MACK, H.: Jour. A. Ph.A. 27: 632, 1938.
5. BISCHOFF, F., AND LONG, M. L.: Amer. J. Cancer 32: 418, 1938.
6. TANNENBAUM, A.: "Approaches to Tumor Chemotherapy". Publication of the A.A.A.S., 1947, p. 96.
7. TANNENBAUM, A.: Personal communication.

TOXICOLOGICAL INVESTIGATIONS OF COMPOUNDS PROPOSED FOR USE AS INSECT REPELLENTS¹

A. LOCAL AND SYSTEMIC EFFECTS FOLLOWING TOPICAL SKIN APPLICATION

JOHN H. DRAIZE, ELSIE ALVAREZ, AND MARIE F. WHITESELL

B. ACUTE ORAL TOXICITY

GEOFFREY WOODARD AND E. CONWAY HAGAN

C. PATHOLOGICAL EXAMINATION

ARTHUR A. NELSON

*Division of Pharmacology, Food and Drug Administration, Federal Security Agency
Washington 25, D. C.*

Received for publication December 8, 1947

INTRODUCTION. Although insect pests and insect-borne diseases have plagued man through the ages, intensified research in the field of insect repellents is a relatively recent undertaking. Prior to World War II the most commonly used repellents were relatively ineffective substances such as citronella, oil of cloves, cinnamon, lemon, peppermint, camphor, pennyroyal and a few synthetic organic compounds such as terpinyl acetate, salicylic acid esters and ethylene glycol.

In 1940 Granett (1, 2) as a result of his entomological tests on more than one thousand chemical compounds or mixtures thereof listed nine conditions which an ideal insect repellent should satisfy. As a result of these researches, Granett found a mixture, which he called "Sta-Way," to meet most of the conditions listed. The only reference to irritation and toxicity stipulated in Granett's list of conditions for an ideal repellent is "It must be non-irritating to the skin and harmless when breathed or accidentally swallowed." The possibility of the percutaneous absorption of the repellent or of irritation following repeated daily use over a period of time was not considered. The effect of repeated application proved Sta-Way to be an undesirable repellent. Repellents which may be well tolerated following a few applications may, as reported by Hoehn (3), become severely damaging following continued liberal use. Because of the inherent dangers from the use of repellents, the Surgeon General of the U. S. Army asked the U. S. Public Health Service and the Food and Drug Administration to conduct tests on proposed insect repellent candidates in order to establish their safety for use by the military personnel.

METHOD. A. *General Remarks.* Approximately 4000 compounds, prepared by various universities and chemical concerns, were submitted during the war program to the Food and Drug Administration for toxicological testing. Most of the compounds were organic chemicals; however, a few mixtures also were submitted. Ten thousand rabbits were used to determine the primary irritation and skin toxicity of these insect repellent candidates.

¹ A portion of the funds used in this investigation was supplied by a transfer recommended by the Office of Scientific Research and Development and the War Department to the Division of Pharmacology of the Food and Drug Administration.

The toxicological technics employed were described by Draize et al (4). During the course of these studies many compounds did not survive the initial screening procedures because of excessive skin irritation or because of systemic toxicity following initial exposure. Many of the compounds which passed the "screening for irritation" tests were eliminated in acute and 3-week subacute skin toxicity testing. In the final group surviving the initial tests there remained 42 compounds or mixtures which were studied intensively. Ninety-day subacute toxicity tests were employed whereby a series of rabbits were inuncted daily with the insect repellent at varying dosage levels on a weight basis.

B. Remarks on Acute Oral Toxicity. As will be observed in the table, the acute oral LD 50's for 39 of the 42 compounds were determined on from one to five species of animals for each compound. Except where noted otherwise, the materials were given undiluted. The rats, guinea pigs, and chicks were routinely fasted for 18 hours prior to administering the chemical, while the mice and rabbits were not fasted. The animals were observed for six days following dosage, and deaths occurring in that period were recorded. The materials were given in graded doses to groups of ten animals each so that dosage-mortality curves could be constructed. The LD 50's were then estimated from these curves.

C. Remarks on Pathological Examinations. The portion of the table dealing with the pathological examination is based on material from 441 rabbits started on 90-day subacute inunction experiments only. Rabbits from other types of studies of these compounds are not included. About 7000 individual pieces of tissue were examined. It is obvious that the results must be given in a highly condensed form to allow inclusion in the table.

The lesions listed in the table are those which were present in a number or degree beyond the so-called spontaneous lesions seen in control animals. Where a gradation of a lesion is given (e.g., from moderate to slight) it means that the more marked lesions were seen at the higher dosage levels, and vice versa.

Changes resulting from inanition (atrophy of spleen, liver, fat, etc.) have usually not been listed, but "inanition" listed instead. It has often been difficult to allocate atrophy of the testis between inanition and the agent inuncted, but where atrophy of the testis is mentioned, it is thought to be in major part a direct toxic effect of the agent used.

Rabbits dying during the first 72 hours of the experimental period were usually not sent to the pathological laboratory. The same was true for some rabbits dying on week ends and showing extensive autolysis when found. With few exceptions the remaining animals were examined grossly and microscopically.

Non-surviving rabbits, and survivors on the higher dosage levels, routinely had hematoxylin-eosin stained paraffin sections of formalin-fixed tissue made from heart, liver, gall bladder, spleen, pancreas, mesenteric lymph nodes, kidney, adrenal, thyroid, testis (or uterus and ovary), bone marrow, thigh muscle, skin, and four levels of brain. In addition, frozen sections of liver and kidney were stained for fat with an oil-soluble red dye, and a Giemsa-stained smear of bone marrow was made. Parathyroid was generally sectioned incidentally with the thyroid. Other structures were sectioned when indicated by gross examination; these were most frequently gastrointestinal tract and lung. Bouin and Zenker fixations

TABLE 1

CHEMICAL NAME	ACUTE ORAL TOXICITY		SKIN TOXICITY		LOCAL SKIN REACTION	PATHOLOGY	CONCLUSIONS
	Species and No. Used	Est'd LD 50	Acute Est'd LD 50	Subacute (90-day) LD 50			
		mg.	ml./kg.	ml./kg.			
Alpha, alpha' di-methyl al- pha carbobutoxy-dihydro- gamma pyrone (Indalone) (O-9) ¹	Rabbits (80)	5.4	10+	2	Slight dermatitis in rabbits. Very mild erythema in the copiously perspiring hu- man. (5.3) ¹	Focal necrosis of gall bladder and upper gastrointestinal tract, and of hepatic and renal tubular cells. ¹ In survivors, possibly slight protein leakage in kidney, and slight hepatitis. Der- matitis +++ to +++ ++ to +	In single or acute dosage—little or no effects. Repetition of high doses causes moderately severe skin re- actions. Skin which regenerated af- ter initial injury was more resistant to the compound than original skin. Nonirritating to the conjunctiva of rabbits. Poorly absorbed by skin. Safe for restricted use.
	Mice (100)	11.6					
	G. pigs (110)	3.2					
	Chicks (80)	15.0					
	Rats (70)	7.4					
2-ethyl hexano-diol 1,3 (Rutgers 612) (O-375)	G. pigs (40)	1.9	10+	2	Moderate dermatitis in rab- bits. No dermatitis in man. (4.3)	Moderate to slight hydropic degeneration of hepatic and renal cells; moderate tubular atrophy of testis. In survivors, no lesions. Dermatitis +++ to +++ +.	Poorly absorbed by skin. Small amounts absorbed produce a nar- coleptic. Skin lesions noted in doses as low as 0.5 ml./kg. in rabbits fol- lowing multiple dose applications. Regenerated skin is more resistant to action of "612". Severely toxic upon absorption; considered safe for recommended use.
	Chicks (40)	1.4					
	Mice (150)	4.2					
	Rats (90)	2.5					
Dimethyl phthalate (NTM) (O-262)	G. pigs (80)	2.4	10+	4+	No skin irritation. Irritat- ing to mucous membrane. (0.7)	Pulmonary edema; slight kidney damage. In sur- vivors, varying degrees of nephritis at two higher levels; lower two levels negative. Dermatitis ab- sent.	Very poorly absorbed by the skin. Mild symptoms of systemic effects at highest dosage level employed (injections of 4.0 ml./kg./day). Safe for recommended use.
	Mice (110)	7.2					
	Rats (40)	6.0					
	Rabbits (80)	4.4					
	Chicks (120)	8.5					
A mixture of following com- position: Dimethyl phthalate—8 parts "R-612"—2-ethyl hexanedio- l 1,3—2 parts Indalone—2 parts (O-1473)	G. pigs (50)	2.8	10+	2-	Slight dermatitis in rabbits. Mild erythema on repeated application in the human. (3.8)	Inanition; moderate hyper- plasia of bone marrow; slight kidney damage. In survivors, mild changes of inanition. Dermatitis ++++ to +++ ±.	Very poorly absorbed by the skin. Produces skin changes slightly less severe but of same nature as that produced by "Indalone" and "R- 612". It is somewhat more toxic than either "R-612" or Indalone when used in comparable quanti- ties. Animals survived only 7 to 8 daily injections of 4 ml./kg. Safe for recommended use.
	Rabbits (40)	2.4					
	Rats (50)	5.9					
	Mice (80)	9.2					

Diethylene glycol mono-butyl ether acetate (BCA) (O-170)	Rabbits (100) G. pigs (50) Mice (100) Chicks (50)	2.8 2.7 7.1 0.6 5.0	5.5	2.0	No gross skin irritation (2.1)	Hematuria; hemolysis in kidneys; renal tubular degenerative changes; inanition. In survivors, slight residual kidney lesions; inconsistently other changes. Dermatitis \pm ; +.	Toxicity of BCA is increased when applied to skin in combination with agent such as corn oil. Readily absorbed by intact as well as abraded skin. Causes hematuria and extensive in vivo hemolysis. Hemoglobinuria severe at 4.0 ml./kg. Unsafe for use.
Sta-Way Insect Repellent "PCA" (diethylene glycol monobutyl ether acetate)—50% Diethylene glycol monoethyl ether (Carbitol)—15% Ethyl alcohol—25% Corn oil—7% Oil of lavender (1 oz. to gal.) (O-171)	Oral toxicity data obtained on individual components—not on completed formulation.		5.0 \pm	2.0	No gross skin irritation. (1.3)	Hematuria; hemolysis in kidneys; renal tubular degenerative changes; inanition. In survivors, slight residual kidney lesions; inconsistently other minor changes. Dermatitis dehydrating; \pm .	Larger rabbits (body weight 3.0+ kg.) are more susceptible than those below 2.5 kg. of body weight. The mixture is readily absorbed by the skin and in doses above 2.0 ml./kg./day causes in vivo hemolysis, internal organ damage and hemoglobinuria. Unsafe for use.
N-butyl d,l malate (R-69) (O-337)	Mice (50)	20.0	10+	1.0-2.0	Slight to moderate gross skin irritation following multiple dose applications. (4.4)	Focal myocarditis; hepatitis; voluntary muscle necrosis; hemorrhages in gastrointestinal tract; inanition; hyperplasia of bone marrow. In survivors, not consistent. (Only two lower levels survived.) Dermatitis necrotizing; ++.	1 ml./kg. represents the limit of tolerance. In cases of fatal poisoning, there is severe skin irritation, anoxia and body weight losses. Unsafe for use.
2,4 Dinitroanisole (SAT) Treated as a 5% solution in dimethyl phthalate for acute work. 3% solution in propylene glycol for subacute testing. (O-184)	No information		—	120 mg./kg.	Patch testing of wetted crystals produced no irritation of the skin of rabbits. 3.0 (5% solution in carbitol)	(?) Pneumonia. In survivors, no lesions. Dermatitis \pm ; +.	A 3% solution of 2,4 dinitroanisole in propylene glycol produces no primary irritation. A slight eczematoid reaction on hands of an operator handling the solution daily during a 13-week period. A 3% solution in propylene glycol safe for recommended use.
Isopropyl cinnamate (ELEY) (O-3026)	G. pigs (50)	2.7	>10+	4.0+	Severe skin damage from repeated daily application of 2.0 or 4.0 ml./kg. (5.3)	Not characteristic. In survivors, atrophy of testis, hyperplasia of bone marrow and slight changes of inanition at one or two highest levels. Dermatitis +++ to ++++; +.4-4.4. In +.	Thick crusting of skin in ratio to size of dose administered. Moderate chronic dermatitis. Although not very toxic it produces rather severe skin injury. It would be contraindicated for use on hyperirritable or sensitive skin.

TABLE 1—Continued

CHEMICAL NAME	ACUTE ORAL TOXICITY		SKIN TOXICITY		LOCAL SKIN REACTION	PATHOLOGY	CONCLUSIONS
	Species and No. Used	Est'd LD 50 mg./kg.	Acute Est'd LD 50 ml./kg.	Subacute (90-day) LD 50 ml./kg.			
n-Propyl cinnamate (ELET) (O-2024)	G. pigs (50) Mice (50)	3.0 7.0	—	2.0	Mildly irritating to skin following repeated exposure. (3.8)	Inanition; various inconsistent acute lesions. In survivors, moderate atrophy of testis; inconsistently slight bone marrow hyperplasia and minimal epinitis. Dermatitis necrotizing; ++.	This compound is more toxic but less irritating to the skin than isopropyl cinnamate (O-2029). No cumulative effects were observed. At three lower dose levels no significant abnormalities in internal organs. Safe within restricted conditions of use.
para-Methoxy benzyl alcohol (SSML) (O-1170)	Mice (50) Rats (50)	1.0 1.2	10+	1-2	Moderately irritating on repeated application especially to abraded skin. Skin reactions are sharply defined on basis of dose administered. (4.0)	In survivors, no lesions. Dermatitis; +.	The limit of tolerance is sharply defined and is between 1.0 and 2.0 ml./kg. In fatalities severe depression and dyspnoea prior to death. Unsafe for use.
2-phenyl cyclohexanol (ESNN) (O-2133)	Rats (50) Mice (50) Rabbits (40) G. pigs (50)	3.5 5.4 2.7 1.0	10+	2	No gross skin irritation on first exposure but moderate dermatitis develops following multiple dose exposure. (2.3)	In survivors, hyperplasia of bone marrow and testicular atrophy proportional to dosage level. Dermatitis ++++; +++++.	The compound produces severe skin lesions at all levels after repeated applications. Toxicity except for the local skin reactions was not observed at and below levels of 2.0 ml./kg. Unsafe for use.
2-Phenoxy ethylacetate (ESTB) (O-2145)	Rats (50) Mice (70)	4.9 3.7	9.0	4.0—	No gross skin irritation on first exposure; very mild or no local skin reactions upon repeated applications. (0.2)	Hematuria; hemolysis in kidneys; necrosis in liver and stomach and elsewhere; fatty degeneration in liver and kidney. In survivors, possible slight decrease in m/e ⁺ ratio in bone marrow. Dermatitis absent; ±.	Fatally poisoned subjects exhibit hematuria. Toxicologically the compound behaves in "all-or-none" manner with 4.0 ml./kg. being critical dose level. When this level is exceeded there is extensive kidney damage, however, safe for recommended use.

sec butyl phthalimide (ET-31) (O 210)	G pigs Rabbits Rats Mice	(40) (60) (40) (70)	12 23 11 10	10+	2-4	No gross skin irritation following repeated application (1.9)	Inanition, moderate hyperplasia of bone marrow. In survivors, no lesions. Dermatitis +, +	Although no deaths occurred from acute (single dose) skin exposure at doses of 0.5 ml/kg there was evidence of systemic effects, e.g. anorexia, depression and weakness in subjects. At dose levels of 2.0 ml/kg and lower there was no evidence of systemic effects during 90 days of treatment. Safe for recommended use.
p-propyl phenyl methyl alcohol (SM-17) (O 174)	Mice Rats	(100) (70)	30 18	60	10+	Slight skin irritation from initial exposure. Mild to moderate skin irritation at higher dose levels following repeated applications (2.1)	Varicose slight acute lesions. In survivors, renal tubular calcification in two of five, acute calcification in one of these. Dermatitis necrotizing, ++	Moderately toxic following acute (single) skin exposure to relatively large doses. Two operators were sensitized by handling this compound. Produced death in animals from relatively low doses (1.0 ml/kg) in 4 daily injections. Severe depression leading to moribund condition some time prior to death. Unsafe for recommended use.
Piperonyl cyclohexanone (Activator 312) (Tested as 5% solution in NTM) (O 2518)	Rats Mice	(80) (70)	60 51	—	<20 (of 5% soln in NTM)	Very mild skin irritation following repeated daily applications 1.8 (5% solution in dimethyl phthalate)	Slight to moderate acute lesions in several locations. In survivors, increased incidence of "spontaneous" encephalitis and nephritis (90 vs 15-20%), moderate atrophy of testis, two each of focal myocardial necrosis, hepatitis, splenitis. Dermatitis +, +	Daily application of doses of 2.0 ml/kg produced severe poisoning. Very little skin damage was noted. The limited use of 5% solutions of Activator 312 in dimethyl phthalate is considered safe.
Benzyl benzoate (BLN) (O 523)	Rats Rabbits Mice G pigs	(40) (30) (50) (60)	17 18 14 10	40	20	Very mild gross skin irritation (7.8)	Inanition. In survivors, atrophy of testis at two higher levels, possibly increased incidence of focal nephritis and encephalitis. Dermatitis +, +	Benzyl benzoate acts as an "all or none" toxicant. A sharp demarcation is approached at dosage levels of approximately 2.0 ml/kg/day. Delayed deaths following acute (large single) skin exposures. Animals die without exhibiting prior symptoms of systemic effects. Safe for use on a restricted basis as in scabicides.

TABLE 1—Continued

CHEMICAL NAME	ACUTE ORAL TOXICITY		SKIN TOXICITY		LOCAL SKIN REACTION	PATHOLOGY	CONCLUSIONS			
	Species and No. Used	Est'd LD 50	Acute Est'd LD 50	Subacute (90-day) LD 50						
		ml./kg.	ml./kg.	ml./kg.						
Di-isopropyl tartaric acid (NDME) (O-3572)	Rats (50)	1.0	10.0+	4.0--	Nonirritating to intact skin. Mildly irritating to abraded skin. (3.5)	Various necrotizing lesions (one animal). In survivors, slight hepatic cell necrosis at two higher levels; giant regenerating hepatic cells; (?) atrophy of testis, increased incidence of "spontaneous" nephritis and encephalitis, slight reduction of m/e ratio in bone marrow. Dermatitis, patchy necrosis (one animal); + to 0.	Poorly absorbed by the skin. No symptoms of toxicity in single (acute) exposures at doses of 0.4 ml./kg. In multiple dose administration, there is initial poisoning followed by apparent recovery. Safe for recommended use.			
	Mice (60)	1.4								
2-phenylethyl alpha hydroxy isobutyrate (SYBL) (O-1650)	Mice (50)	6.3	10.0+	4.0+	Nonirritating to skin. (1.4)	All survived. Little if any damage; possible slight tendency to increase in "spontaneous" nephritis and encephalitis, and slight atrophy of testis. Dermatitis + to 0.	Poorly absorbed by either intact or abraded skin. In acute exposures no gross symptoms of poisoning except a polyuria. Safe for recommended use.			
	Rats (20)	6.0								
	Rabbits (50)	1.0	6.0	1.0+				Mildly irritating to the skin. (2.9)	Severe kidney damage (one animal). In survivors, slight hepatic cell necrosis; (?) slight excess of focal nephritis and encephalitis. Dermatitis ++; ++ to +.	Poorly absorbed by either intact or abraded skin. Poisoned subjects exhibit varying degrees of anorexia. Fatal cases exhibit severe depression prior to death. Unsafe for recommended use.
	G. pigs (60)	1.1								
1-(p-methoxy phenyl)-2-methyl 1,3-propanediol methylene ether (O-5533)	Mice (70)	2.8			Mild gross skin irritation. (2.3)	Not characteristic. In survivors, little if any damage; no consistent lesions. Dermatitis ±; ++ to +.	Poorly absorbed by either intact or abraded skin. Highest tolerated dose without symptoms or systemic effects is 1.0 ml./kg./day. At 2.0 ml./kg. and higher, severe anorexia and body weight loss. Unsafe under conditions of recommended use.			
	Rats (50)	3.3								
	Rats (50)	4.2	10+	4.0--						
	Mice (50)	4.5								

Pentamethylene dipropionate (1,3-pentanediol dipropionate) (O-4154)	Rats Mice	(50) (50)	9.1 10.4	4.0+	4.0+	Very mild skin irritation. (5.3)	All survived. Atrophy of testis at two higher levels; little otherwise. Dermatitis + to 0.	Readily absorbed by the skin. The slope of dosage-mortality curve is quite flat. In fatal cases, death is preceded by severe anorexia and depression. Safe for use.
Ethyl cyanocyclohexyl acetate (O-7021)	Rats Mice	(50) (50)	6.4 5.0	2.0+	10.0+	Moderate gross skin irritation. (7.9)	Inanition; acute duodenitis. In survivors, emaciation at 4 ml./level; some atrophy of testis; little otherwise. Dermatitis +++ to ++;	Not readily absorbed by the skin. No deaths following acute exposures (3.9-9.4 ml./kg.) but all animals exhibited symptoms of poisoning. In 90-day subacute (inunction) experiments, poisoning was observed at dose levels of 1.0 ml./kg./day. Limited use with caution permissible.
Ethanol-2,2'-thiodi-, diacetate (O-5342)	Rats Mice	(50) (40)	8.2 7.2	4.0+	10.0+	Mildly irritating only following massive dosages. (1.5)	Inanition (one animal). In survivors, no lesions. Dermatitis 0; + to 0.	Absorbed readily by either intact or abraded skin. With the exception of one rabbit at 4.0 ml./kg. the entire group survived the 90-day inunction period without evidence of gross systemic effects. Safe for recommended use.
1,2,3,4 tetrahydro-2-naphthol (K-801) (O-5563)	Rabbits G. pigs Rats Mice	(50) (30) (50) (50)	2.8 1.0 1.0 2.0	1.0+	4.0+	Moderate to severe gross irritation producing a red-leathery appearance of the skin. (8.5)	Centrolubular necrosis of liver; focal hemorrhagic necrosis in stomach and elsewhere; pulmonary suppuration. In survivors, hyperplasia of bone marrow; minimal hepatic cell necrosis; slight hepatitis. Dermatitis necrotizing; + to +++.	Poorly absorbed from acute massive exposure, but with production of moderate skin irritation. Slope of dosage-mortality curve is quite flat. Recoveries and deaths occurring at dose levels ranging from 3.3 to 9.4 ml./kg. In 90-day experiments the limit of tolerance is approximately 1.0 ml./kg./day. Unsafe for unrestricted use. (Small quantities may be used with caution.)
Ethyl ester of epoxy methyl phenyl acrylic acid (O-7020)	Rats Mice	(50) (40)	0.1 5.6	Flat slope of dosage mortality curve 1.0+	9.0	Moderately irritating in large acute exposures. (2.4)	Inanition; little otherwise. In survivors, no lesions. Dermatitis +++; + to +.	Slope of dosage-mortality curve is quite flat, deaths occurring from daily inunctions of doses as low as 0.5 ml./kg./day. Unsafe under conditions of recommended use. (Small quantities may be used with caution.)
N,N-Diethyl succinamide n-propyl ester (O-6163)	Mice Rats	(70) (40)	0.6 0.3	4.0+	9.0	Moderately irritating from large (acute) exposures. (2.4)	Not characteristic. In survivors, no lesions. Dermatitis +++; +.	Readily absorbed by the skin. Safe under recommended conditions of use.

TABLE 1—Continued

CHEMICAL NAME	ACUTE ORAL TOXICITY		SKIN TOXICITY		LOCAL SKIN REACTION	PATHOLOGY	CONCLUSIONS
	Species and No. Used	Inst'd. LD 50	Acute Est'd. LD 50	Subacute (90-day) LD 50			
		ml./kg.	ml./kg.	ml./kg.			
p-(n-propoxy) benzaldehyde (O-5518)	Rats (70) Mice (90)	1.6 1.8	9.0	1.0+	In large acute exposures there is severe gross skin irritation. (1.7)	Not characteristic (two animals). In survivors, no lesions. Dermatitis +++; ++.	Readily absorbed by the skin, and is relatively toxic upon repeated application. Limit of tolerance to repeated application is 1.0 ml./kg./day. Unsafe under recommended conditions of use.
Hexahydrophthalic acid diethyl ester (O-5557)	Rats (40) Mice (60)	3.9 2.4	10.0+	4.0+	Very mild irritation only from large acute exposures. (1.5)	Inanition; noncharacteristic lesions (two animals). In survivors, emaciation at two higher levels; some hyperplasia of bone marrow; little otherwise. Dermatitis ++ to +; ++ to +.	Poorly absorbed by the skin. Marked emaciation in all rabbits at 4.0 ml./kg. surviving entire 90-day period. Safe for recommended use.
Cyclohexyl acetate (O-6230)	Mice (70)	7.2	10.0+	4.0+	No gross skin irritation from large single (acute) exposures. Very mild skin irritation from repeated exposures. (2.3)	Inanition; noncharacteristic lesions (two animals). In survivors, slight focal myocarditis; moderate atrophy of testis; slight increase in incidence of focal nephritis. Dermatitis ++ to +; ++ to +.	Readily absorbed by the skin. Little or no skin irritation. Safe for recommended use.
2-nitro-2-methyl 1,3 propanediol (Solid liquefies with gentle heat) (O-7102)	Mice (100) Rats (80) (50% in corn oil)	6.3 4.0	10.0+	2.0+	No irritation by single large (acute) exposures; very mild irritation following repeated applications especially at two higher dose levels (2.0 and 4.0 ml./kg./day). (1.4)	Focal myocarditis; slight centrolubular necrosis of liver; protein leakage in kidneys; left shift in bone marrow; inanition. In survivors, lesions as given for non-survivors inconspicuous or absent, especially at two lower levels; reduction of m/o ratio in bone marrow. Dermatitis +++	Poorly absorbed, solidifies at skin temperatures. Unsafe for recommended use.

Hydroxylic acid B-phenyl-ethyl ester (O-6216)	Rats Mice	(40) (50)	7.8 4.6	10.0	4.0+	No irritation from single large (acute) exposures nor from repeated applications (1.6)	All survived. Slight fatty and/or hydropic degeneration of liver; moderate to slight atrophy of testis. Dermatitis + to 0.	This compound is one of the least toxic following skin application of the group in this report. Safe for recommended use.
Acetophenone 4-methoxy 3-methyl (O-10516)	Mice Rats	(70) (70)	3.6 1.5	6-8	2.0+	In single large (acute) exposure the compound produces moderate to severe gross skin irritation. (4.4)	Inanition; little otherwise. In survivors, inanition at highest (2 ml.) level; little otherwise. Dermatitis +++ to ++; +++ to ++.	Readily absorbed by the skin. Although no deaths occurred, symptoms of poisoning occurred at dose levels of 0.5 and 1.0 ml./kg./day. Depression with severe anorexia occurred at higher dose levels, as well as moderate gross skin irritation at these higher dose levels (2.0 and 4.0 ml./kg./day). Unsafe for recommended use.
Cyclohexane carboxylic acid, 1-hydroxy cyclopentyl ester (O-6133)	Mice Rats	(80) (60)	3.2 2.0	9.4+	1.0-	Mildly irritating following large single (acute) exposures. (3.0)	Not extensive in either survivors or non-survivors. Dermatitis +++ to ++; ++ to +.	Readily absorbed by the skin. Slight systemic effects following single (acute) exposures to 9.4 ml./kg. Moderate to severe skin irritation developed following repeated applications. Compound is quickly fatal. Unsafe for recommended use.
N-butyl 1,2,3,6-tetrahydronaphthalimide (O-7146)	Mice Rats	(80) (100)	3.3 2.5	10.0+	0.5-	Mildly irritating to skin. (1.8)	Necrosis of renal papilla and ureteral wall; hyperplasia of transitional epithelium of urinary tract. Dermatitis +++; no survivors.	Poorly absorbed by the skin from single (acute) exposures. Produces severe hematuria in subjects following repeated application. Severe urinary tract damage. Unsafe for recommended use.
N-N Dipropyl succinamic acid ethyl ester (O-6232)	Mice Rats	(90) (50)	3.6 0.2	10.0+	1.0-	Mildly irritating upon first exposure; repeated applications produce severe gross skin irritation. (1.7)	Inanition; focal hemorrhagic necrosis stomach-duodenum; moderate kidney lesions (various); slight hyperplasia bone marrow. In survivors, slight hyperplasia of bone marrow; possibly some testicular atrophy. Dermatitis ++; ++.	Poorly absorbed by skin following single (acute) exposures. In repeated exposures subjects at 0.3 ml./kg./day (lowest dosage level employed) exhibited much damage. Unsafe for recommended use.

TABLE 1—Concluded

CHEMICAL NAME	ACUTE ORAL TOXICITY		SKIN TOXICITY		LOCAL SKIN REACTION	PATHOLOGY	CONCLUSIONS
	Species and No. Used	Est'd. LD 50	Acute Est'd. LD 50	Subacute (90-day) LD 50			
		ml./kg.	ml./kg.	ml./kg.			
2-nitro-2-ethyl-1,3-propanediol butyraldehyde acetal (O-7090)	Rats (60) Mice (60)	2.0 3.1	9.4	2.0+	Mild gross skin irritation from either single or multiple dose applications. (1.3)	Not characteristic (two animals). In survivors, lesions few and not consistent nor characteristic. Dermatitis +; +.	Poorly absorbed by skin. Dose levels of 2.0 ml./kg./day produced severe emaciation. Unsafe for recommended use. Infrequent or restricted use permissible.
Mixture of: 2-phenyl cyclohexanol (O-2133)—70 parts 1,2,3,4-tetrahydro-2-naphthol (O-3553)—30 parts (NMRI 201) ^a (O-8604)	Mice (60) Rats (60)	2.1 1.4	—	2.0+	Moderate to severe skin irritation. (6.7)	Not extensive. In survivors, slight liver damage (focal necrosis; hepatitis); slight hyperplasia of bone marrow. Dermatitis necrotizing; +++ to ++.	Severe to moderate skin irritation following low repeated dosage levels of 0.5 ml./kg./day. Limit of tolerance approximately 1.0 ml./kg./day. Unsafe for recommended use. Infrequent or limited use in conditions of dry (nonperspiring) skin is permissible.
Mixture of: 2-phenyl cyclohexanol (O-2133)—70 parts 2-cyclohexyl cyclohexanol (O-1023)—30 parts (NMRI 449) ^a (O-9327)	G. pigs (60) Mice (60) Rats (60)	1.7 5.8 3.5	—	2-4	Severe skin damage after daily repetition of dose over 90-day period.	Necrosis of adrenal cortex and bone marrow. In survivors, not great; in general present with higher dosage levels and not with lower; in emulsion group marked to moderate atrophy of testis, moderate to slight hyperplasia of bone marrow, and minor changes in spleen and thyroid; in pure compound group slight hyperplasia of bone marrow, slight splenitis, and possibly slight atrophy of testis. Dermatitis necrotizing; +++ to ++.	Dry, leathery (parchmentlike) skins. Roughening of skin with eschar formation on doses as low as 0.5 ml./kg./day. Unsafe for recommended use. Infrequent or limited use in condition of dry skin permissible.
Mixture of: 2-phenyl cyclohexanol (O-2133)—35 parts 2-cyclohexyl cyclohexanol (O-1023)—15 parts Water + emulsifier q.s.—100 parts	G. pigs (80) Mice (60) Rats (80)	2.8 13.9 7.8	—	4.0+			Although the toxicity in this preparation and in O-9327 is proportional to the percentage of O-2133 and O-1023 in the formula, severe skin irritation leading to eschar formation was obtained in doses of 0.5 ml./kg. of the emulsion. Unsafe for recommended use. Infrequent or limited use on dry skin is permissible.

Piperonyl ether butoxide (piperonyl butoxide) (33-1-17) (O-14250)	Rats Mice	11.5 8.3	—	4.0 (of the 5% solution)	No skin irritation observed. 1.0 (5% solution in dimethyl phthalate)	5/5 survivors, slight reduction of m/s ratio in bone marrow. Dermatitis: 0.	The 5% solution of O-14250 in dimethyl phthalate produces no skin irritation. Safe for recommended use.
Polyalkylene glycol stearate (Stearate 61 C-2250) Treated as 2% aqueous emulsion (O-2453)	No information.	—	—	In excess of 4.0 ml./kg. of 3% aqueous emulsion.	Moderate skin irritation leading to feed of infection. (2.3)	5/5 survivors, moderate to slight liver damage (hepatic cell hydrops and necrosis); slight hyperplasia of bone marrow; possibly some atrophy of testis. Dermatitis: + + + to + +.	Daily applications of 2.3% of aqueous emulsion produced a devitalization of skin and many foci of skin infections. Permissible for infrequent use as insecticide, houseflies, etc.

Figures and letters in this column refer to the Orlando Code.

Numerical values in this column represent the scores of Indices of Primary Irritation.

First sentence under "Pathology" for each compound gives visceral lesions in non-surviving animals.

+ + + = marked; + + = moderate; + = slight; ± = questionable; 0 = none. Values for dermatitis before semicolon refer to non-survivors, those following to survivors.

All four non-survivors died within the first few days and were therefore not sent to the pathology laboratory.

One animal; four others died within the first few days and were therefore not sent to the pathology laboratory.

m/s = myeloid/erythroid.

Code name given by Naval Research Medical Institute.

One animal, not examined microscopically.

were occasionally used. Routine sections for surviving animals on the lower dosage levels included heart, liver, gall bladder, kidney, testis (or uterus and ovary), bone marrow (plus smear) and skin; other structures were sectioned as indicated by gross examination or by the findings in the previous category of animals.

DISCUSSION. The toxicological testing of insect repellents for their local and systemic effects was undertaken for the armed services in the recent war program. The requirements and conditions of use for insect repellents by military personnel differ from those for use by the civilian population. The military personnel is composed mainly of young adults under close medical supervision, whereas potential civilian users would include the very young, the aged, and the infirm. The repellents classified "deemed safe for recommended use" in the table were adjudged safe for military personnel under the conditions of use required in their operations. Among the numerous requirements for military personnel was the use of the repellent under tropical conditions, that is, heavy perspiration, and application to large areas of the body surface. Under such conditions it was calculated that the individual might have to use daily applications of approximately 0.25 ml./kg. Therefore, these requirements stipulated for military use are more rigorous than might be obtained in a temperate climate such as that of the continental United States.

The primary consideration in the physiological effects of insect repellents is the toxicity and irritation following skin application or the wearing of repellent-impregnated clothing. Since there is the danger of accidental swallowing, acute oral LD 50 figures are included in the table. Comparison of LD 50 figures for acute oral administration and acute topical skin application indicates no direct relationship in toxicity by these two routes of administration. Systemic effects resulting from topical skin administration depend not only on the inherent toxicity of the preparation but also on the ease of absorption of the material by the skin.

Chemical structure and potential skin absorption may not be correlated since compounds closely related in chemical structure often exhibit widely different orders of absorbency by the skin. However, in the measure of primary skin irritation, compounds with similar structure sometime exhibit similar degrees of irritancy. Free acids, amines, aldehydes and ketones, especially those with lower molecular weights, have generally been severely irritating. A number of exceptions, however, were encountered. Such exceptions were sufficiently numerous so that potential skin irritation may not be predicted solely on a basis of chemical structure.

In the course of the toxicological investigations, it became apparent that the longer subacute experiments, involving daily application to the skin of relatively small doses over a period of 90 days, were indispensable for the proper toxicological appraisal of a preparation. Compounds which may be tolerated well by acute (single) or by multiple application for a relatively short period of time may become severely damaging if daily applications are continued for a 90-day period.

In preparations containing a number of components it is necessary to test the

completed formulation, since the resultant toxicity of the latter may be greater than the combined toxicity of the component parts, that is, the resulting toxicity may exhibit potentiation rather than simple addition.

In the final toxicological appraisal of a preparation, the following data in addition to LD 50 and the percutaneous toxicity and irritation are considered—(a) toxicity by other routes of administration; (b) histopathology of all 90-day animals, including those severely poisoned, but surviving four or more days of treatment; (c) hematology; (d) chemical studies on blood and urine, and (e) distribution of compound in tissues (whenever analytical procedures permit). These data are utilized for an evaluation of the extent of injury, and for an estimation of its likelihood of repair. After allowances are made for animal species differences and for a margin of safety, a final conclusion as to the safety of frequent application by man of a given repellent may be rendered.

CONCLUSION

Extensive data on skin and oral toxicology, histopathology, hematology and clinical chemistry of blood and urine are summarized for animals treated with each of the 42 insect repellents. Conclusions were drawn as to their safety for use by military personnel. Eighteen of these preparations were deemed safe for use whereas 24 did not pass the rigorous testing procedure and appraisal.

REFERENCES

- (1) GRANETT, P.: Jour. Econ. Entom. 33: 563, 1940.
- (2) GRANETT, P.: Jour. Econ. Entom. 33: 566, 1940.
- (3) HOEHN, D.: Jour. Amer. Med. Assoc. 128: 513, 1945.
- (4) DRAIZE, J. H., WOODARD, G., AND CALVERT, H. O.: THIS JOURNAL 82: 377, 1944.

LOCUS OF THE ADRENERGIC BLOCKING ACTION OF DIBENAMINE¹

MARK NICKERSON AND GEORGE M. NOMAGUCHI

Department of Pharmacology, University of Utah School of Medicine, Salt Lake City, Utah

Received for publication December 16, 1947

The recent demonstration of the high potency and specificity of the adrenergic blocking action of N,N-dibenzyl- β -chloroethyl amine (Dibenamine) (1, 2) raises the question of its locus and mode of action. Data presented previously (2) have ruled out any major effect on autonomic ganglia or postganglionic neurones or on the release, alteration or destruction of epinephrine and sympathin. Figure 1 illustrates most of these points. Here the nicotinic reaction to carbachol in an atropinized cat treated with Dibenamine illustrates the normal reactivity of the sympathetic ganglia and adrenal medulla. Although the pressor effect of the sympatho-adrenal discharge is reversed by Dibenamine, the vascular response persists for almost exactly the same period of time as before, indicating a comparable course of release and destruction of epinephrine and sympathin. The same conclusion is suggested by the fact that Dibenamine does not markedly alter the sinus tachycardia induced by epinephrine (3) or circulating sympathin. *In vitro* experiments were also reported as evidence for the absence of any direct interaction between epinephrine and Dibenamine. The liberation of sympathin E after treatment with Dibenamine was shown by the delayed tachycardia which occurs in adrenalectomized animals after stimulation of the peripheral end of the severed splanchnic nerves.

The above data indicate that the blocking action of Dibenamine is exerted directly upon effector cells (vascular smooth muscle in the cases considered). This action could conceivably be exerted by preventing the penetration of sympathomimetic agents to their site of action, by blocking or destroying some receptor substance or mechanism necessary for excitation by these agents, or by attacking the contractile mechanism itself. The experiments presented below were undertaken to distinguish between these possibilities.

Effect on Permeability. The suggestion that adrenergic blocking agents may act by altering the permeability of effector cells to epinephrine has been made in connection with 933F, yohimbine and other agents (4). The primary consideration in this interpretation is that the agents in question much more readily block responses to circulating epinephrine than those to sympathetic nerve stimulation. It is assumed that the chemical mediator is released within the effector cells in the latter case.

Dibenamine also blocks the effects of circulating epinephrine more readily than the effects of sympathetic nerve stimulation, as shown in figure 2. Here the

¹ This investigation was aided by a grant from Givaudan-Delawanna, Inc. The Dibenamine used in these experiments was kindly supplied by Dr. William Gump of this company.

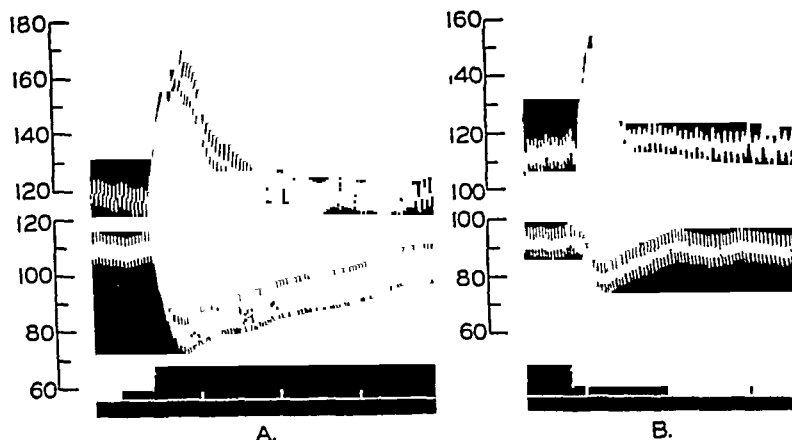


FIG. 1. BLOOD PRESSURE RESPONSES TO THE INTRAVENOUS ADMINISTRATION OF 150 μ GM./KGM. OF CARBACHOL IN ATROPINIZED CATS UNDER PENTOBARBITAL ANESTHESIA

A. Upper record before and lower record after the intravenous administration of 15 mgm./kgm. of Dibenamine. Injection of carbachol indicated by arrow. Time in minutes. Ordinate, mm. Hg.

B. The same procedures as in A after bilateral adrenalectomy.

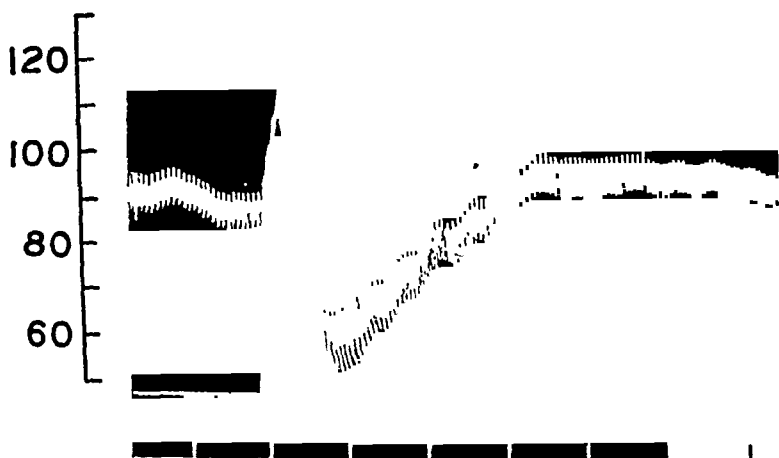


FIG. 2

Blood pressure response of cat under urethane anesthesia to electrical stimulation of the peripheral ends of the severed right splanchnic nerves after incomplete adrenergic block with Dibenamine. Period of stimulation indicated by upward deflection of signal line. Time in minutes. Ordinate, mm. Hg.

immediate effect of splanchnic nerve stimulation is unaltered although the later action of endogenously released epinephrine is reversed. Figure 1B illustrates that large doses of Dibenamine are capable of reversing the blood pressure response to generalized sympathetic nerve stimulation even in the absence of the adrenals. However, both excitatory and inhibitory reactions to sympathin are involved in this response, and because only the algebraic sum of the effects can be recorded, an accurate analysis is impossible.

The nictitating membrane of the cat is a more favorable structure upon which to study the quantitative aspects of Dibenamine action. The smooth muscle of this organ is innervated entirely by sympathetic fibers and responds to sympathetic stimulation only by contraction. In addition, only a fraction of the smooth muscle fibers are directly innervated (4, 5) and consequently it is possible to study the effects of Dibenamine both upon directly innervated smooth muscle fibers and upon non-innervated fibers which receive sympathin by diffusion from adjacent cells. Dibenamine markedly reduces, but frequently does not completely abolish, the response of the nictitating membrane to cervical sympathetic stimulation (2), and consequently it is possible to analyze the quantitative aspects of this blocking action.

Responses of the nictitating membrane to electrical stimulation of the homolateral cervical sympathetic nerve before and after treatment with Dibenamine were studied in cats under urethane or pentobarbital anesthesia. No significant difference in results with the two types of anesthesia was noted, except that fewer eye-ball movements occurred when urethane was employed and consequently smoother and somewhat more accurate baselines were obtained. Shielded platinum electrodes were applied to the homolateral cervical sympathetic nerve which was severed proximal to the electrodes. Intermittent, rectilinear pulses of 0.5 millisecond duration and of variable intensity and frequency were employed, using an electronic stimulator whose output current is largely independent of the resistance in the external circuit. In a few experiments, stimuli were applied to the postganglionic fibers but inasmuch as this procedure gave results comparable to preganglionic stimulation, the latter was selected for routine use because it is simpler and permits more reproducible placing of the electrodes. Contractions of the nictitating membrane were recorded on a smoked drum by a frontal writing lever which amplified the response sevenfold. In all cases, the data are recorded as millimeters movement of the writing lever. Contraction of the nictitating membrane was induced by 5-second periods of stimulation at a rate of 20 pulses/second with gradually increasing intensity. The smallest current which gave the maximal response under these conditions was employed for studies of the effects of various rates and durations of stimulation.

If Dibenamine were acting to decrease the permeability of effector cells to sympathomimetic amines it should block the contraction of those cells which are excited by sympathin diffusing from the outside, but not the contraction of directly innervated cells. Therefore, it should inhibit a definite percentage of the maximum response. The extent of this block would be quantitatively dependent upon the ratio of innervated to non-innervated cells.

Figure 3 shows the distribution of percentages of the maximum control response to cervical sympathetic stimulation remaining after treatment with 40 mgm./kgm. Dibenamine. Only records from animals which remained in good condition during the entire experiment (as indicated by a mean carotid blood pressure greater than 70 mm. Hg) are included. It is clear from the data presented that there is no constant reduction in the response. Results with equal doses of the inactive alcohol derivative of Dibenamine (dibenzyl-ethanol amine) and with doses of pentobarbital which reduced the arterial pressure to 60 mm. Hg or less are included to demonstrate that the observed reduction in response is due to the specific adrenergic blocking activity of Dibenamine.

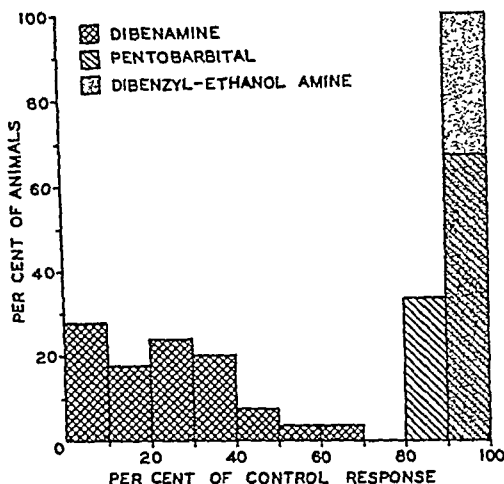


Fig. 3

Distribution of the degree of reduction of the maximum nictitating membrane response to cervical sympathetic nerve stimulation in a group of 38 animals treated with Dibenamine (40 mgm./kgm.) or with control substances.

Sixteen animals were tested after two different dose levels of Dibenamine. In this group, the average residual response was 38% after 20 mgm./kgm. and 20% after a total of 40 mgm./kgm. Dibenamine, indicating a progressive reduction in response. Inasmuch as 20 mgm./kgm. Dibenamine completely blocks the nictitating membrane response to circulating epinephrine (see below), the further reduction in response after a total of 40 mgm./kgm. weighs against the possibility that Dibenamine acts only by preventing penetration of the effector cells by sympathomimetic agents.

Additional information was obtained from a study of the relative effectiveness of Dibenamine in blocking contraction of the nictitating membrane induced by various frequencies and durations of cervical sympathetic stimulation. The release of small amounts of sympathin (short duration or low frequency of stimulation) should provide a relatively greater stimulus to directly innervated as

immediate effect of splanchnic nerve stimulation is unaltered although the later action of endogenously released epinephrine is reversed. Figure 1B illustrates that large doses of Dibenamine are capable of reversing the blood pressure response to generalized sympathetic nerve stimulation even in the absence of the adrenals. However, both excitatory and inhibitory reactions to sympathin are involved in this response, and because only the algebraic sum of the effects can be recorded, an accurate analysis is impossible.

The nictitating membrane of the cat is a more favorable structure upon which to study the quantitative aspects of Dibenamine action. The smooth muscle of this organ is innervated entirely by sympathetic fibers and responds to sympathetic stimulation only by contraction. In addition, only a fraction of the smooth muscle fibers are directly innervated (4, 5) and consequently it is possible to study the effects of Dibenamine both upon directly innervated smooth muscle fibers and upon non-innervated fibers which receive sympathin by diffusion from adjacent cells. Dibenamine markedly reduces, but frequently does not completely abolish, the response of the nictitating membrane to cervical sympathetic stimulation (2), and consequently it is possible to analyze the quantitative aspects of this blocking action.

Responses of the nictitating membrane to electrical stimulation of the homolateral cervical sympathetic nerve before and after treatment with Dibenamine were studied in cats under urethane or pentobarbital anesthesia. No significant difference in results with the two types of anesthesia was noted, except that fewer eye-ball movements occurred when urethane was employed and consequently smoother and somewhat more accurate baselines were obtained. Shielded platinum electrodes were applied to the homolateral cervical sympathetic nerve which was severed proximal to the electrodes. Intermittent, rectilinear pulses of 0.5 millisecond duration and of variable intensity and frequency were employed, using an electronic stimulator whose output current is largely independent of the resistance in the external circuit. In a few experiments, stimuli were applied to the postganglionic fibers but inasmuch as this procedure gave results comparable to preganglionic stimulation, the latter was selected for routine use because it is simpler and permits more reproducible placing of the electrodes. Contractions of the nictitating membrane were recorded on a smoked drum by a frontal writing lever which amplified the response sevenfold. In all cases, the data are recorded as millimeters movement of the writing lever. Contraction of the nictitating membrane was induced by 5-second periods of stimulation at a rate of 20 pulses/second with gradually increasing intensity. The smallest current which gave the maximal response under these conditions was employed for studies of the effects of various rates and durations of stimulation.

If Dibenamine were acting to decrease the permeability of effector cells to sympathomimetic amines it should block the contraction of those cells which are excited by sympathin diffusing from the outside, but not the contraction of directly innervated cells. Therefore, it should inhibit a definite percentage of the maximum response. The extent of this block would be quantitatively dependent upon the ratio of innervated to non-innervated cells.

Figure 3 shows the distribution of percentages of the maximum control response to cervical sympathetic stimulation remaining after treatment with 40 mgm./kgm. Dibenamine. Only records from animals which remained in good condition during the entire experiment (as indicated by a mean carotid blood pressure greater than 70 mm. Hg) are included. It is clear from the data presented that there is no constant reduction in the response. Results with equal doses of the inactive alcohol derivative of Dibenamine (dibenzyl-ethanol amine) and with doses of pentobarbital which reduced the arterial pressure to 60 mm. Hg or less are included to demonstrate that the observed reduction in response is due to the specific adrenergic blocking activity of Dibenamine.

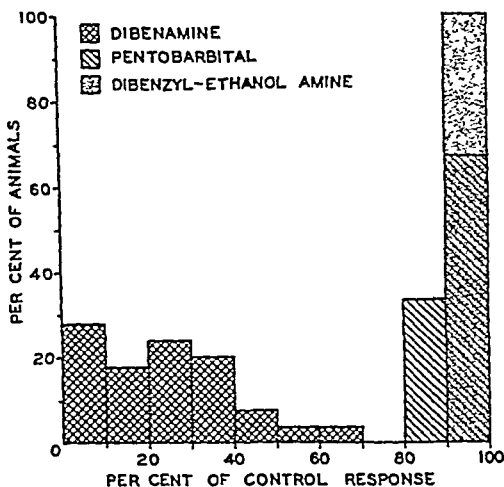


FIG. 3

Distribution of the degree of reduction of the maximum nictitating membrane response to cervical sympathetic nerve stimulation in a group of 38 animals treated with Dibenamine (40 mgm./kgm.) or with control substances.

Sixteen animals were tested after two different dose levels of Dibenamine. In this group, the average residual response was 38% after 20 mgm./kgm. and 20% after a total of 40 mgm./kgm. Dibenamine, indicating a progressive reduction in response. Inasmuch as 20 mgm./kgm. Dibenamine completely blocks the nictitating membrane response to circulating epinephrine (see below), the further reduction in response after a total of 40 mgm./kgm. weighs against the possibility that Dibenamine acts only by preventing penetration of the effector cells by sympathomimetic agents.

Additional information was obtained from a study of the relative effectiveness of Dibenamine in blocking contraction of the nictitating membrane induced by various frequencies and durations of cervical sympathetic stimulation. The release of small amounts of sympathin (short duration or low frequency of stimulation) should provide a relatively greater stimulus to directly innervated as

compared to non-innervated cells, since the latter receive sympathin by diffusion only after a concentration gradient has been established by the accumulation of mediator in innervated cells. Nerve stimulation adequate to produce a maximal

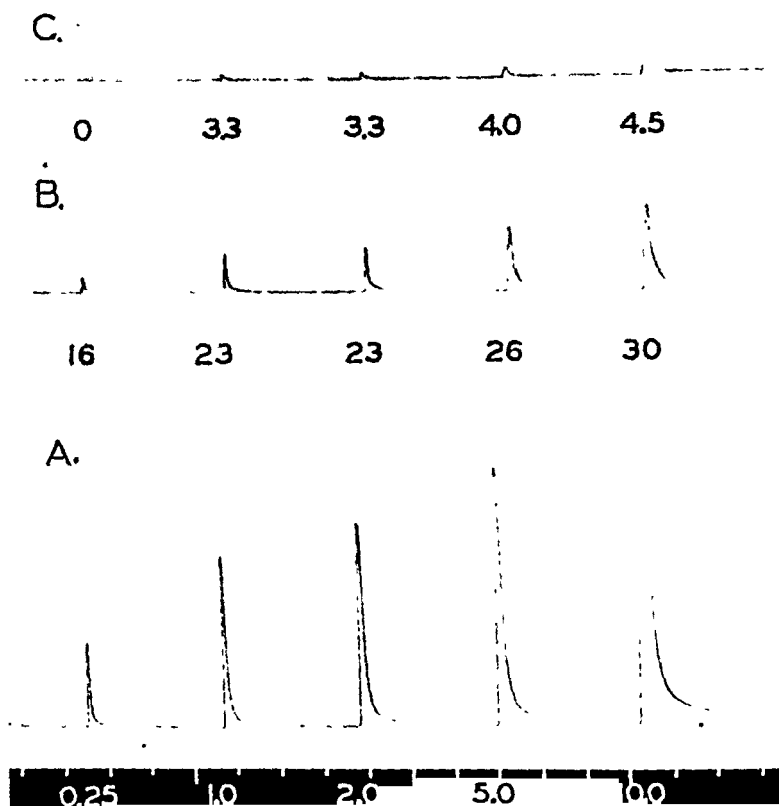


FIG. 4. A SERIES OF RECORDS SHOWING THE RESPONSE OF THE NICTITATING MEMBRANE TO VARIOUS PERIODS OF MAXIMAL SYMPATHETIC NERVE STIMULATION

A. Control.

B. 45 minutes after intravenous injection of 20 mgm./kgm. of Dibenamine.

C. 45 minutes after an additional 20 mgm./kgm. of Dibenamine.

Lowest row of figures indicates duration of stimulus in seconds.

Upper two rows of figures give per cent of control response to the same stimulus. Stimuli at 3 minute intervals. Time in minutes.

response activates all contractile cells. A blocking agent acting by altering permeability would therefore be expected to inhibit a larger percentage of the response to maximal stimulation than of that to stimulation of short duration or low frequency.

The exact opposite of this situation was found to occur after Dibenamine

block. The response to a short (1.0 sec.) stimulus was reduced more than that to a long (10 sec.) stimulus with the same characteristics (20 pulses/sec. and maximal intensity). The average residual response was 15% for the short and 29% for the long stimuli. This difference is statistically significant. The percentage residual response to the two types of stimulation for each of 11 animals was treated as a ratio, which should have been equal to one if the responses to the two periods of stimulation were equally reduced. The mean of these ratios proved to be 0.49 ± 0.062 . Since responses to stimulation are reproducible to less than $\pm 5\%$ by the technic employed, this value differs from unity by 6.5 times the standard error of the difference and is therefore highly significant.



FIG. 5

Response of nictitating membrane to 10-second periods of maximal stimulation (indicated by white bar) of the cervical sympathetic nerve as recorded on a fast moving paper. Upper curve shows control response, middle curve shows response 45 min. after intravenous administration of 20 mgm./kgm. Dibenamine and lower curve shows response 45 min. after an additional 20 mgm./kgm. Dibenamine. Time in 2-second intervals.

Figure 4 illustrates the type of records obtained in these experiments. The same effect is seen in figure 5 which shows responses to 10-second periods of stimulation recorded on a faster moving paper. It will be noted that after Dibenamine the latent period of the contraction is much increased and contraction during the first second of stimulation is particularly reduced.

In figure 6 are plotted the results of a similar experiment in which the duration and intensity of stimulation remained constant and the frequency was varied between 0.5 and 120/second. After Dibenamine, the response to low frequencies is relatively more reduced than that to high frequencies. Stimulation with frequencies up to 10/second elicited no detectable response after large doses of Dibenamine although prior to treatment responses up to 80% of the maximum were produced. Klopp (6) found that diffusion of sympathin to non-innervated

cells affected the contraction of the cat's nictitating membrane only at stimulation frequencies above 4 to 14/second (the exact frequency varied in different individuals). Therefore, the complete elimination by Dibenamine of responses to low frequencies of stimulation must be due to inhibition of the responses of directly innervated cells.

If the Dibenamine block were due to a decreased permeability of effector cells it should be possible to overcome this block by sufficiently increasing the con-

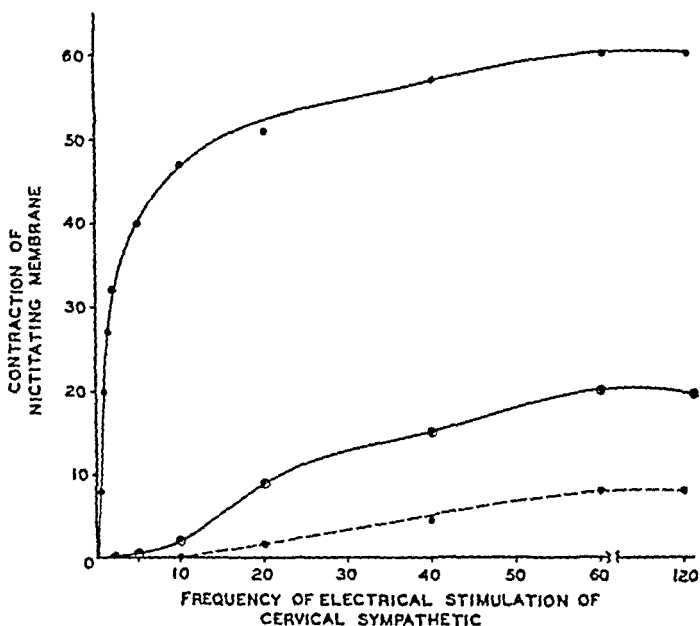


FIG. 6

Plot of contraction of nictitating membrane against frequency of electrical stimulation of cervical sympathetic nerve. All stimuli 5 seconds in duration. Ordinate, mm. movement of writing lever; abscissa, pulses/second.

●—● Control values.

○—○ 45 minutes after 20 mgm./kgm. of Dibenamine given intravenously.

●- - -● 45 minutes after an additional 20 mgm./kgm. of Dibenamine.

centration gradient. However, it is not possible to overcome the Dibenamine block of the nictitating membrane either with maximal sympathetic stimulation or massive doses of epinephrine (fig. 7).

It was previously shown (2) that Dibenamine does not interfere with the response of smooth muscle cells which respond to epinephrine by relaxation. This may be considered as additional proof that Dibenamine does not act by altering the permeability of effector cells. It is difficult to conceive of a situation in which the permeability of smooth muscle cells responding to epinephrine by contraction is markedly decreased while the permeability of similar cells responding by relaxation is unaltered by the same blocking agent.

Effect on Contractile Mechanism. A second possible locus of action of Dibenamine is on the contractile mechanism of smooth muscle cells. This mode of action might offer an explanation for the ability of the drug to block excitatory but not inhibitory effects of epinephrine. Simeone and Sarnoff (7) have suggested that Dibenamine may act as a generalized tissue poison on the basis of its local destructive action after subcutaneous or intraperitoneal injection (2). This possibility was investigated by a study of the effect of Dibenamine on the

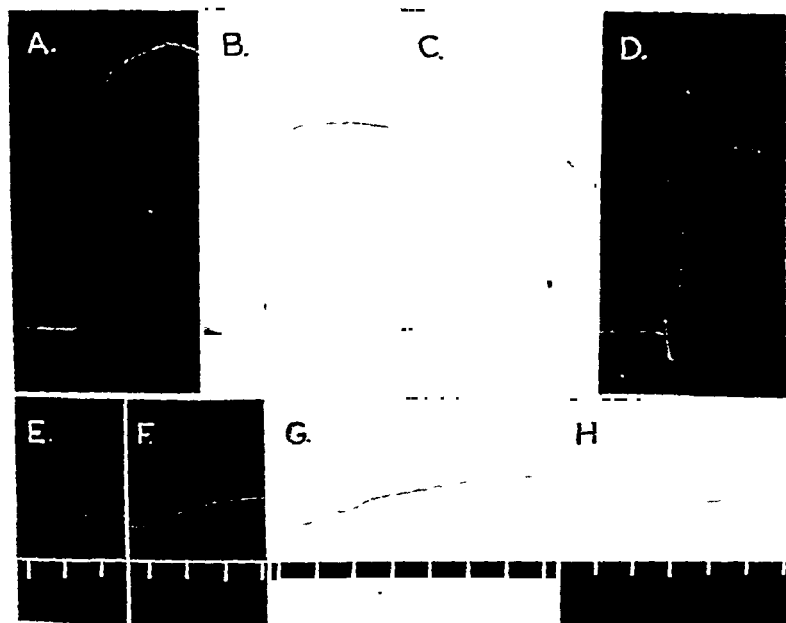


FIG. 7

Response of chronically denervated nictitating membrane to various substances administered intravenously before (A to C) and after (D to H) the intravenous administration of 20 mgm./kgm. Dibenamine.

A. 2.5 μ gm./kgm. nor-epinephrine.

B. 2.5 μ gm./kgm. epinephrine.

C. 10 μ gm./kgm. methacholine.

D. 10 μ gm./kgm. methacholine.

E. 2.5 μ gm./kgm. nor-epinephrine.

F. 2.5 μ gm./kgm. epinephrine.

G. 50 μ gm./kgm. epinephrine.

H. 1000 μ gm./kgm. epinephrine.

response of the chronically (8-20 days) denervated nictitating membrane to various agents. In all cases the adrenals were removed to eliminate possible effects of endogenously released epinephrine.

Figure 7 illustrates a typical series of responses. In all cases the contraction in response to epinephrine and to nor-epinephrine² (suggested by Euler [8] and Gaddum and Goodwin [9] to be sympathin E) is abolished even when the doses are increased 1000 and 100 times, respectively. The slow rise of the base-line

² Kindly supplied by Dr. M. L. Tainter of the Sterling-Winthrop Research Institute

cells affected the contraction of the cat's nictitating membrane only at stimulation frequencies above 4 to 14/second (the exact frequency varied in different individuals). Therefore, the complete elimination by Dibenamine of responses to low frequencies of stimulation must be due to inhibition of the responses of directly innervated cells.

If the Dibenamine block were due to a decreased permeability of effector cells it should be possible to overcome this block by sufficiently increasing the con-

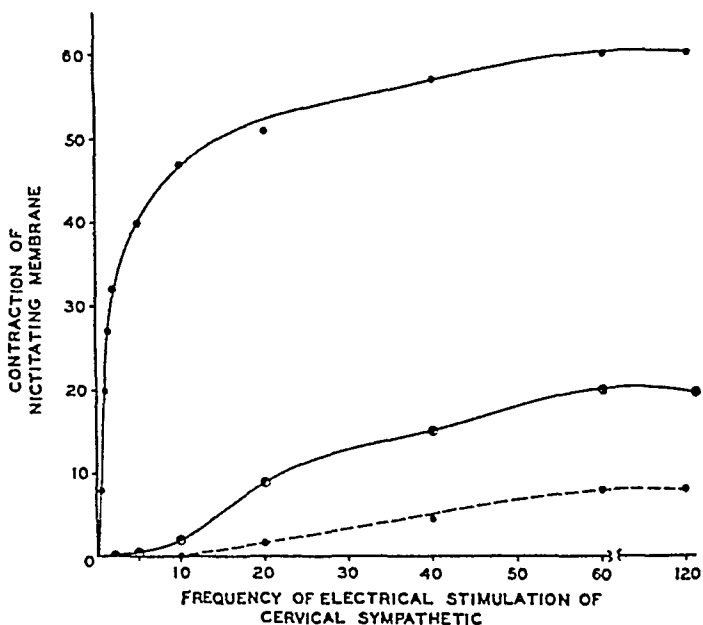


Fig. 6

Plot of contraction of nictitating membrane against frequency of electrical stimulation of cervical sympathetic nerve. All stimuli 5 seconds in duration. Ordinate, mm. movement of writing lever; abscissa, pulses/second.

- Control values.
- 45 minutes after 20 mgm./kgm. of Dibenamine given intravenously.
- 45 minutes after an additional 20 mgm./kgm. of Dibenamine.

centration gradient. However, it is not possible to overcome the Dibenamine block of the nictitating membrane either with maximal sympathetic stimulation or massive doses of epinephrine (fig. 7).

It was previously shown (2) that Dibenamine does not interfere with the response of smooth muscle cells which respond to epinephrine by relaxation. This may be considered as additional proof that Dibenamine does not act by altering the permeability of effector cells. It is difficult to conceive of a situation in which the permeability of smooth muscle cells responding to epinephrine by contraction is markedly decreased while the permeability of similar cells responding by relaxation is unaltered by the same blocking agent.

Effect on Contractile Mechanism. A second possible locus of action of Dibenamine is on the contractile mechanism of smooth muscle cells. This mode of action might offer an explanation for the ability of the drug to block excitatory but not inhibitory effects of epinephrine. Simeone and Sarnoff (7) have suggested that Dibenamine may act as a generalized tissue poison on the basis of its local destructive action after subcutaneous or intraperitoneal injection (2). This possibility was investigated by a study of the effect of Dibenamine on the

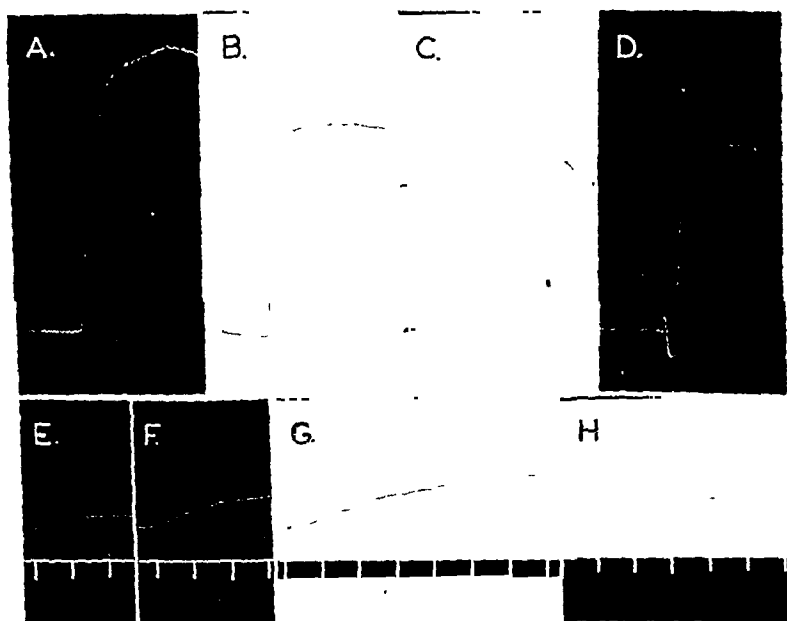


FIG. 7

Response of chronically denervated nictitating membrane to various substances administered intravenously before (A to C) and after (D to H) the intravenous administration of 20 mgm./kgm. Dibenamine.

A. 2.5 μ gm./kgm. nor-epinephrine.

B. 2.5 μ gm./kgm. epinephrine.

C. 10 μ gm./kgm. methacholine.

D. 10 μ gm./kgm. methacholine.

E. 2.5 μ gm./kgm. nor-epinephrine.

F. 2.5 μ gm./kgm. epinephrine.

G. 50 μ gm./kgm. epinephrine.

H. 1000 μ gm./kgm. epinephrine.

response of the chronically (8-20 days) denervated nictitating membrane to various agents. In all cases the adrenals were removed to eliminate possible effects of endogenously released epinephrine.

Figure 7 illustrates a typical series of responses. In all cases the contraction in response to epinephrine and to nor-epinephrine² (suggested by Euler [8] and Gaddum and Goodwin [9] to be sympathin E) is abolished even when the doses are increased 1000 and 100 times, respectively. The slow rise of the base-line

² Kindly supplied by Dr. M. L. Tainter of the Sterling-Winthrop Research Institute.

seen when epinephrine or nor-epinephrine is administered after Dibenamine is apparently secondary to vascular changes in the orbit and is unrelated to dosage.

In contrast to its effective block of responses to sympathomimetic drugs, Dibenamine has little or no effect upon the contraction induced by methacholine (fig. 7). Dibenamine does cause a partial reduction in the response to histamine; but this is not surprising inasmuch as Dibenamine has a weak antihistaminic action, and closely related compounds are potent antihistaminics (Nickerson and Bullock, 10). The above experiments indicate that the contractility of smooth muscle cells of the nictitating membrane is not impaired by doses of Dibenamine adequate to block completely the response to epinephrine.

DISCUSSION. By a process of elimination it may be concluded that Dibenamine acts by blocking some essential step in the excitation process occurring between the penetration of sympathomimetic agents to their site of action and the actual contraction of the smooth muscle cell. Presumably, this step involves a specific combination of the exciting agent with some cellular constituent. Almost nothing is known of the nature of the combination or of the "receptor substance" involved. However, this reaction is of major importance as it apparently accounts for the ability of cells to distinguish between various stimulating agents and thus to exhibit specific responses. In the following discussion the term "receptor substance" is used to designate those cellular factors involved in activation by a specific agent without any implications as to what their nature may be.

It has been found that atropine antagonizes acetylcholine (11), ergotamine antagonizes epinephrine (12) and Benadryl antagonizes histamine (13) at definite ratios of each pair of drugs. The exact ratio is a function of the particular tissue studied. The question of whether this represents the ratio at which the substances in question replace each other in combination with specific receptor substances remains unsettled. Clark (11) has presented certain indirect evidence to indicate that atropine and acetylcholine are not in equilibrium with a common receptor, but the issue is certainly not settled. In any case, these antagonisms have the characteristics of a competitive reaction in that a relatively constant ratio of stimulating and blocking agent will produce a constant reaction over a wide range of concentrations. A response equaling the control value may thus be obtained in the presence of the blocking agent by sufficiently increasing the concentration of the exciting agent.

The action of Dibenamine does not fall into this category since the difference is small between a dose which produces no block of epinephrine or sympathin and one which gives a complete or almost complete block over a very wide range of concentrations. After administration of adequate blocking doses of Dibenamine the control response of the nictitating membrane can never be reproduced or approached even by maximal nerve stimulation or concentrations of epinephrine as large as tolerated by the intact animal.

It is possible that a competitive equilibrium between Dibenamine and epinephrine or sympathin may exist in the period during which the block is being produced, and this possibility is being investigated. However, after the block has

developed (this may required 30 minutes or more) no such equilibrium is demonstrable. The effect of adequate doses of Dibenamine on the receptor mechanism seems to be overcome only by time. The difference between the action of Dibenamine and other drugs mentioned above may be due to the fact that Dibenamine is the only adequately studied blocking agent which has the potentiality of reacting with various tissue constituents to form stable covalent bonds (Nickerson and Gump, 14). Such a reaction of Dibenamine with a specific receptor substance or grouping might remove the latter rather permanently from participation in reactions with sympathomimetic agents. The block would then be abolished only by dissolution of this bond (perhaps by enzymatic action) or by synthesis of new active groupings. The prolonged block produced by Dibenamine (2) indicates that the restorative mechanism acts slowly.

The ability of Dibenamine to block completely the response of the nictitating membrane to epinephrine but not to nerve stimulation is not easy to explain. It is not simply a matter of difference in chemical configuration between epinephrine and sympathin E because Simeone and Sarnoff (7) have shown that the effect of circulating sympathin E on the denervated nictitating membrane is readily blocked. It is possible that sympathetic stimulation may cause the release of mediator in high local concentrations not duplicated by systemic administration, but this explanation is difficult to check experimentally.

The most likely explanation of the difference between the degree of completeness of the Dibenamine block of responses to epinephrine and of those to sympathetic nerve stimulation rests on a structural basis. It is usually assumed, but has never been demonstrated, that the spatial arrangement of receptors in a particular organ is such that all are equally available for activation. However, it is quite possible that certain contractile cells are relatively inaccessible to substances diffusing from the circulating blood. This relative inaccessibility should apply for both Dibenamine and circulating epinephrine or sympathin and consequently receptors poorly accessible to the blocking action of Dibenamine might also receive only small amounts of circulating sympathomimetic agents. Therefore, the block would appear to be complete. However, there is no reason to believe that poorly vascularized areas are also poorly innervated, and unblocked cells may be stimulated by the release of large amounts of sympathin in their immediate vicinity. If this explanation is correct, the wide variations in the degree to which Dibenamine blocks the responses of individual nictitating membranes to cervical sympathetic nerve stimulation may depend upon rather simple anatomical differences.

After Dibenamine, the recorded response of the nictitating membrane is more sluggish and nearly maximal stimuli are frequently required to produce any detectable contraction. This may be due to the failure of most of the smooth muscle cells to respond. The smooth muscle retractors of the nictitating membrane lie largely outside of the membrane and enter it obliquely for only a short distance (15). Therefore, tension on the center of the marginal cartilage (as applied for recording) is transmitted obliquely across the long axes of the smooth muscle cells and there is no assurance that the smooth muscle mass is under

tension prior to stimulation. Under these conditions part of the contraction may occur before the writing lever is moved. Experiments of Rosenbluth and Cannon (16) on the summation of effects of epinephrine and sympathin indicate that this may be a factor even in the unblocked, sensitized nictitating membrane: It would be a particularly important factor when most of the muscle mass is relaxed and only isolated portions contract. Failure to record the initial portion of the nictitating membrane contraction would account for both the slow response and the greater reduction of the response to weak stimuli after treatment with Dibenamine.

The fact that the apparent degree of block varies markedly with the duration and frequency of stimulation (figs. 4 and 6) makes it difficult to compare the above results with published studies on other blocking agents. However, preliminary observations in our laboratory indicate that large doses (10 mgm./kgm.) of ergotoxine produce a block which is related to frequency and duration of stimulation in much the same way as the Dibenamine block. The failure of large doses of ergotoxine to block completely the nictitating membrane response to nerve stimulation can also be explained on the anatomical basis outlined above, although the picture is complicated by the marked contraction which ergotoxine itself produces.

SUMMARY

Quantitative studies of the inhibition by Dibenamine of the response of the cat nictitating membrane to electrical cervical sympathetic nerve stimulation, and of the response of the chronically denervated nictitating membrane to various sympathomimetic and other agents (histamine, methacholine), have indicated that:

1. Dibenamine does not prevent the free penetration of sympathomimetic agents to their site of action.

2. Dibenamine in blocking doses does not paralyze the contractile mechanism of smooth muscle cells as shown by a normal response to non-sympathomimetic agents.

The mechanism of action of Dibenamine is discussed. Tentative explanations are offered for its long duration of action and for its failure to block completely the response of the nictitating membrane to nerve stimulation despite complete abolition of the response to circulating epinephrine and sympathin.

REFERENCES

1. NICKERSON, M. AND GOODMAN, L. S.: *Proc. Am. Federation Clin. Res.*, 2: 109, 1945.
2. NICKERSON, M. AND GOODMAN, L. S.: *THIS JOURNAL*, 89: 167, 1947.
3. NICKERSON, M. AND SMITH, S. M.: To be published.
4. CANNON, W. B. AND ROSENBLUTH, A.: *Autonomic neuro-effector systems*, The Macmillan Company, New York, 1937.
5. ROSENBLUTH, A. AND RIOCH, D. M.: *Am. J. Physiol.*, 106: 365, 1933.
6. KLOPP, C. T.: *Am. J. Physiol.*, 130: 475, 1940.
7. SIMEONE, F. A. AND SARNOFF, S. J.: *Surgery*, 22: 391, 1947.
8. EULER, U. S. VON: *Acta Physiol. Scand.*, 12: 73, 1946.

9. GADDUM, J. H. AND GOODWIN, L. G.: J. Physiol., 105: 357, 1947.
10. NICKERSON, M. AND BULLOCK, F.: To be published.
11. CLARK, A. J.: J. Physiol., 61: 547, 1926.
12. MENDEZ, R.: THIS JOURNAL, 32: 451, 1928.
13. WELLS, J. A., MORRIS, H. C., BULL, H. B. AND DRAGSTEDT, C. A.: THIS JOURNAL, 85: 122, 1945.
14. NICKERSON, M. AND GUMP, W.: To be published.
15. ACHESON, G. H.: Anat. Rec., 71: 297, 1938.
16. ROSENBLUETH, A. AND CANNON, W. B.: Am. J. Physiol., 99: 398, 1932.

tension prior to stimulation. Under these conditions part of the contraction may occur before the writing lever is moved. Experiments of Rosenblueth and Cannon (16) on the summation of effects of epinephrine and sympathin indicate that this may be a factor even in the unblocked, sensitized nictitating membrane: It would be a particularly important factor when most of the muscle mass is relaxed and only isolated portions contract. Failure to record the initial portion of the nictitating membrane contraction would account for both the slow response and the greater reduction of the response to weak stimuli after treatment with Dibenamine.

The fact that the apparent degree of block varies markedly with the duration and frequency of stimulation (figs. 4 and 6) makes it difficult to compare the above results with published studies on other blocking agents. However, preliminary observations in our laboratory indicate that large doses (10 mgm./kgm.) of ergotoxine produce a block which is related to frequency and duration of stimulation in much the same way as the Dibenamine block. The failure of large doses of ergotoxine to block completely the nictitating membrane response to nerve stimulation can also be explained on the anatomical basis outlined above, although the picture is complicated by the marked contraction which ergotoxine itself produces.

SUMMARY

Quantitative studies of the inhibition by Dibenamine of the response of the cat nictitating membrane to electrical cervical sympathetic nerve stimulation, and of the response of the chronically denervated nictitating membrane to various sympathomimetic and other agents (histamine, methacholine), have indicated that:

1. Dibenamine does not prevent the free penetration of sympathomimetic agents to their site of action.
2. Dibenamine in blocking doses does not paralyze the contractile mechanism of smooth muscle cells as shown by a normal response to non-sympathomimetic agents.

The mechanism of action of Dibenamine is discussed. Tentative explanations are offered for its long duration of action and for its failure to block completely the response of the nictitating membrane to nerve stimulation despite complete abolition of the response to circulating epinephrine and sympathin.

REFERENCES

1. NICKERSON, M. AND GOODMAN, L. S.: *Proc. Am. Federation Clin. Res.*, **2**: 109, 1945.
2. NICKERSON, M. AND GOODMAN, L. S.: *THIS JOURNAL*, **89**: 167, 1947.
3. NICKERSON, M. AND SMITH, S. M.: To be published.
4. CANNON, W. B. AND ROSENBLUETH, A.: *Autonomic neuro-effector systems*, The Macmillan Company, New York, 1937.
5. ROSENBLUETH, A. AND RIOCH, D. M.: *Am. J. Physiol.*, **106**: 365, 1933.
6. KLOPF, C. T.: *Am. J. Physiol.*, **130**: 475, 1940.
7. SIMEONE, F. A. AND SARNOFF, S. J.: *Surgery*, **22**: 391, 1947.
8. EULER, U. S. VON: *Acta Physiol. Scand.*, **12**: 73, 1946.

injections were made in the inferior mesenteric artery through a metal cannula inserted retrograde just distal to the ganglion. The electrical stimuli were square waves or diphasic shocks from a Grass stimulator. Unless otherwise specified below, the stimuli were maximal for the group of fibers being studied.

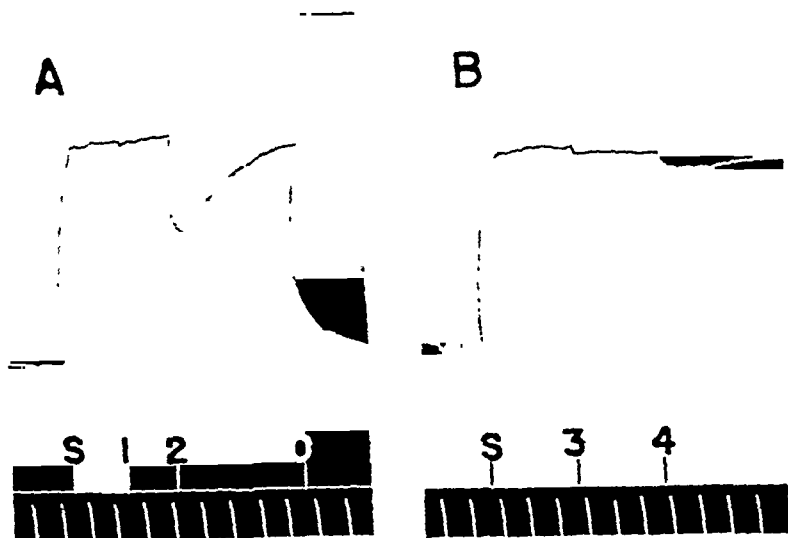


FIG. 1. EFFECT OF MAGNESIUM ION DURING MAXIMAL STIMULATION OF PRE- AND POSTGANGLIONIC FIBERS OF THE SUPERIOR CERVICAL GANGLION

Cat, female, 2.1 kg. Dial anesthesia. Responses of the nictitating membrane.

Preganglionic stimulation in A; postganglionic in B. Stimulus on at S and off at O. At 1, 1 mg. MgSO₄ was injected into lingual artery, at 2, 2.5 mgm., at 3, 2.5 mgm., and at 4, 5 mgm. MgSO₄. Time in 30 seconds.

RESULTS

A. SUPERIOR CERVICAL GANGLION

1. *Ganglionic blockade.* When the nictitating membrane is tonically contracted by maximal stimulation continuously delivered to the cervical sympathetic nerve, injection of magnesium chloride regularly causes relaxation of the membrane. The degree of relaxation is proportional to the dosage. Pronounced relaxations are regularly obtained with 10 to 25 mgm. per kg. given intravenously, 5 to 10 mgm. per kg. given through a fine needle into the carotid, or 1 to 2 mgm. per kg. when delivered through a cannula in the lingual artery with the external carotid tied off distally (Fig. 1, A). When the postganglionic nerves are stimulated magnesium ion often produces a slight relaxation of the nictitating membrane. This is never more than a small fraction of the relaxation obtained from an equal dose injected during preganglionic stimulation (Fig. 1, B). At times no relaxation is seen under such circumstances.

THE BLOCKING ACTION OF MAGNESIUM ION ON SYMPATHETIC GANGLIA¹

JOHN B. STANBURY

Department of Pharmacology, Harvard Medical School

Received for publication December 18, 1947.

The paralyzing action of magnesium ion on skeletal neuromuscular systems has long been recognized (1). As with curare the point of action of magnesium has been located at the neuromuscular junction (2). Curare also inhibits transmission of impulses through autonomic ganglia (3), and recently a similar blockade has been shown to be the principal mode of action of the monovalent quaternary cation tetraethylammonium (4). Indeed the blood pressure lowering effect of the latter drug has been attributed to this effect on the sympathetic ganglia. It was of interest therefore to explore the actions of magnesium ion on autonomic systems in order to see how far the similarity of action of this ion to curare and tetraethylammonium ion might extend, in an endeavor to throw further light on the hypotensive effect of injected magnesium salts. Evidence will be presented to show that magnesium induces a short-lived blockade of sympathetic ganglia, but that its actions differ in several respects from those of curare and tetraethylammonium.

METHODS. Cats were used under anesthesia with 0.7 cc. per kgm. of Dial solution² (each cc. containing diallyl barbituric acid 0.1 gram, urethane 0.4 gram, monoethyl urea 0.4 gram, in aqueous solution) injected intraperitoneally. The methods described by Acheson and Pereira (5) were used in experiments in which pre- and postganglionic fibers of the superior cervical ganglion were stimulated, except that at times the volume of solution injected intra-arterially was varied, and usually a plastic rather than a metal cannula was used in the lingual artery. Injections directly into the carotid artery were made through a 26 gauge needle. Magnesium sulfate was used in a few experiments, but the chloride salt was usually employed. The magnesium chloride and the calcium chloride were made up in 1.05 molar solution (equivalent to 10% $MgCl_2$), and appropriate dilutions were made therefrom. All doses refer to the anhydrous salts. Tetraethylammonium chloride³ was appropriately diluted from a 10% stock solution. Intocostin⁴ was used as a standardized curare preparation. The acetylcholine used was diluted from a 20% stock solution of the bromide salt. Doses refer to the salt. Epinephrine was used as the bitartrate salt. Doses refer to the base.

In the experiments on the stellate ganglion and heart the methods described by Acheson and Moe (4) were used. Heart rates were recorded by the Grass ink-writing oscillograph. Essentially similar methods were used in the experiments on the inferior mesenteric ganglion. The large bowel was retracted laterally with broad ligatures, thereby exposing the ganglion. Stimulating and recording platinum electrodes were appropriately placed, a crush being made between the two recording electrodes to give a monophasic response. A Wagner ground was frequently employed to minimize the stimulus artifact. Intra-arterial

¹ This study was aided in part by a grant from the Milton Fund of Harvard University.

² Generously supplied by Ciba Pharmaceutical Products, Summit, N. J.

³ Generously supplied by Parke, Davis, and Company, Detroit, Michigan.

⁴ Generously supplied by E. R. Squibb and Sons, New Brunswick, N. J.

doses of epinephrine the responses were more nearly the same with and without magnesium ion.

From these results it seems clear that magnesium ion has a blocking action on the transmission of impulses through the superior cervical ganglion. It also has a direct effect on the nictitating membrane not only to cause a further relaxation of the resting membrane, but also to relax the membrane stimulated by nerve impulses or by small amounts of epinephrine. Quantitatively these latter effects are small by comparison with the effect when the nictitating membrane is used as an indicator of ganglionic transmission.

2. *Effect of magnesium ion on ganglionic responses to acetylcholine and potassium ion.* Curarization of autonomic ganglia blocks their response to acetylcholine but not to potassium ion (6). The response of the nictitating membrane to acetylcholine has two components. The initial rapid twitch is due to direct action of the drug on the ganglionic cells, while the prolonged contraction is due to the action of the drug on the membrane itself (7). In our experiments 0.5 mgm. per kg. of atropine sulfate given intravenously had no effect on the quick component, but diminished the slow component of the response.

In three experiments a series of twitches of the nictitating membrane was obtained by injecting acetylcholine bromide intra-arterially into the region of the ganglion. Immediately preceding alternate intra-arterial injections of the drug, varying quantities of magnesium chloride were given through the same cannula. Ten mgm. completely blocked the response of the ganglion to 0.5 mgm. of acetylcholine whether atropine had been given or not (Fig. 2, A). The slow component of the twitch was also eliminated. This dose gave a large but incomplete relaxation of the same membrane when it was contracted by preganglionic stimulation (Fig. 2, B). With smaller doses of magnesium chloride some response to acetylcholine was obtained; the response reached its full height when it was preceded by only 0.65 mgm. The fast component of the contraction of the nictitating membrane disappeared when the ganglion was crushed. The slow component remained (Fig. 2, C).

In the same three experiments uniform twitches of the membrane were obtained by injections of 5 to 10 mgm. of potassium chloride intra-arterially near the ganglion. When the injection was preceded immediately by 5 to 10 mgm. of magnesium chloride no twitches were obtained, but with lesser amounts twitches were obtained, and were of normal height when 0.65 mgm. was injected (Fig. 2, B). The potassium chloride twitches disappeared completely when the ganglion was crushed (Fig. 2, C).

B. STELLATE GANGLION

1. *Effect on the rate of the decentralized heart.* The effect of magnesium ion on the heart rate was explored in 8 cats. In each case both sympathico-vagal trunks were crushed in the neck, the chest was opened, and respiration was maintained artificially. The sympathetic chains on both sides down to and including T4 and all preganglionic fibers to the stellate ganglia were crushed. An electrode for preganglionic stimulation was placed on the communicating branch from the first or second intercostal nerve just distal to the crush. Postganglionic stimula-

In experiments on three cats the action potentials recorded with one electrode on the distal end of the cervical sympathetic nerve and the other on the ganglion were observed during stimulation of the cervical sympathetic nerve proximally. Magnesium chloride injected directly into the common carotid artery caused a sharp fall in the ganglionic potential, and completely eliminated it in doses of 20 to 40 mgm. Recovery was complete in four to seven minutes. The action potential of the preganglionic nerve was not affected by quantities which gave marked falls in the ganglionic potential; with larger doses a slight fall was observed. When submaximal shocks were delivered to the nerve magnesium chloride diminished the action potential considerably.

In experiments on two cats the action potentials of the postganglionic fibers of the superior cervical ganglion were recorded during stimulation of the cervical sympathetic nerve. Injection of magnesium chloride intra-arterially in one case and intravenously in the other was followed by an abrupt fall in amplitude. Complete recovery occurred in 3 to 4 minutes. In one of these experiments it was possible to stimulate the distal end of the ganglion and record farther distally from the tip of the severed end of the postganglionic trunk. There was no diminution in strength of the action current following injection of a quantity of magnesium ion which had a pronounced effect when the stimulus was delivered to the preganglionic fibers. The accessible postganglionic fibers of this ganglion are very short for both stimulation and recording and there was no certainty in this case that the trunk was receiving an adequate blood supply.

A definite but slight relaxation of the nictitating membrane usually occurred following an injection of magnesium ion when the ganglion had been crushed and no stimulus was being applied. Sodium chloride injected in concentrations of 4.5 to 15% while the membrane was resting, or was contracted under postganglionic stimulation, usually caused a similar relaxation of the membrane, but always much smaller than when magnesium chloride was given under the same conditions in equivalent or more dilute solutions. This indicates that the action of magnesium ion was not an osmotic effect. A similar slight relaxation of the unstimulated membrane was usually obtained after removal of the eyeball, or after curarization of the animal with Intocostrin so that strong stimulation of the sciatic caused no response of the muscles of the leg, or when both these procedures were carried out. Hence the action of magnesium ion on the unstimulated membrane could not be attributed to an effect on the skeletal muscle of the orbit.

In order to investigate further the direct effect of magnesium ion on the membrane itself dose response curves to epinephrine alone and epinephrine with added magnesium chloride were obtained on nine cats. In each case the response to a given dose of epinephrine was compared with the response to the same dose of epinephrine in the same volume but containing 20 to 40 mgm. of magnesium chloride. In 4 of these experiments the animal was curarized, in a fifth the eyeball was removed and the animal curarized, and in another the eyeball was removed but no curare was given. The injections were made directly into the carotid artery. When the dose of epinephrine was less than one microgram, magnesium ion lessened or obliterated the small response, whereas with larger

ing electrodes were applied to the fibers which run medially from the ganglion to join the vagus trunk and which then descend to the heart. The heart was thus severed from its neural connection with the central nervous system. In all cases but one the adrenal glands were tied off. In two of these experiments

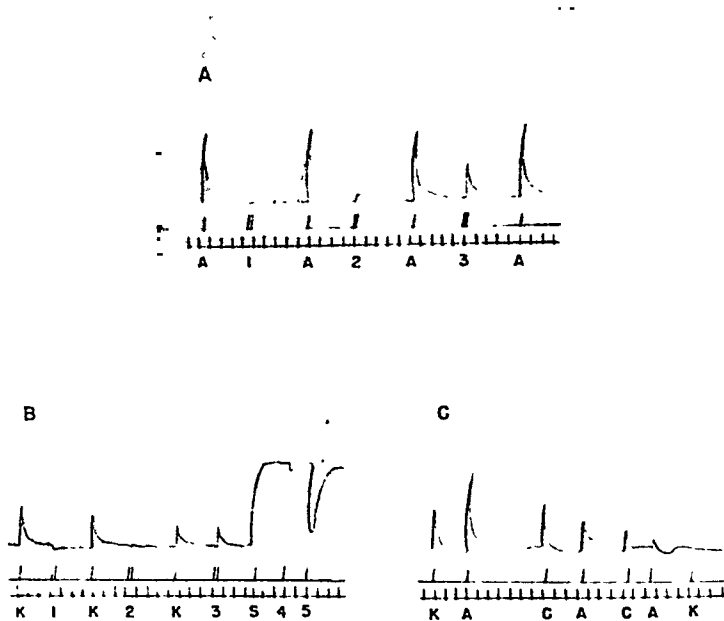


FIG. 2. EFFECT OF MAGNESIUM ION ON RESPONSES OF SUPERIOR CERVICAL GANGLION TO ACETYLCHOLINE AND POTASSIUM ION

Cat, female, 2.2 kg. Dial anesthesia. Atropine sulfate 0.5 mg./kg. All injections of $MgCl_2$ given in 1 cc. Time in 30 seconds. Recording of nictitating membrane.

A. Acetylcholine and magnesium ion. At A, 0.5 mgm. acetylcholine bromide. At 1, 10 mgm. $MgCl_2$ followed by 0.5 mgm. acetylcholine. At 2, 5 mgm. $MgCl_2$ followed by same dose of acetylcholine. At 3, 2.5 mgm. $MgCl_2$ followed by same dose of acetylcholine.

B. Potassium ion and magnesium ion. At K, 10 mgm. KCl. At 1, 10 mgm. $MgCl_2$ followed by same dose of KCl. At 2, 5 mgm. $MgCl_2$ followed by same dose of KCl. At 3, 2.5 mgm. $MgCl_2$ followed by same dose of KCl. At S, maximal stimulation of cervical sympathetic nerve. At 4, 2.5 mgm. $MgCl_2$. At 5, 10 mgm. $MgCl_2$.

C. Influence of crushing of ganglion. Ganglion crushed at C and C. At K, 10 mgm. KCl. At A, 0.5 mgm. acetylcholine bromide.

the right vagus was severed a week previously high in the neck to allow degeneration and thereby eliminate the possibility of spread from the stimulating electrodes and consequent interference from vagal effects. The results were not different in these animals from those obtained in the others.

Intravenous magnesium chloride slowed the unstimulated decentralized heart, the degree of slowing being dependent on the dosage (Fig. 3). In one experi-

observed when the stimulus was applied to the distal end of the crushed ganglion (Fig. 4, B). Recovery was prompt, requiring usually no more than three to five minutes. This quick recovery is in contrast to the delayed or incomplete return of heart rate to the base line following magnesium ion during stimulation of the accelerator nerve, and indicates that the slowing is not entirely dependent upon an effect on sympathetic tone.

C. INFERIOR MESENTERIC GANGLION

In five cats the effect of magnesium chloride was observed on transmission through the inferior mesenteric ganglion. In four of these the injection was made intravenously and in one retrograde through the inferior mesenteric artery. The action potentials were recorded from electrodes placed on the hypogastric nerve 2 to 3 cm. beyond the ganglion. If maximal shocks were delivered to the preganglionic fibers and magnesium ion was injected the amplitudes of the recorded action potentials were sharply reduced proportional to the quantity injected (Fig. 4, C). Ten mgm. per kg. always produced a striking change. The same effect was demonstrable with much smaller doses if given intra-arterially into the region of the ganglion.

When the postganglionic fibers (hypogastric nerve) were stimulated, there was no appreciable change in the action potential when maximal shocks were used (Fig. 4, D). As in the cervical sympathetic nerve during submaximal stimulation magnesium ion reduced the amplitude of the action potential. This indicated that the circulation of the nerve trunks in the region of the stimulating electrodes was intact, and that magnesium ion increased the threshold of these nerve fibers. Once all the fibers were stimulated magnesium ion in the quantities used here did not interfere with the propagation of the nerve impulse.

D. INFLUENCE OF MAGNESIUM ION ON VAGAL INHIBITION

Experiments were done on four cats with decentralized hearts to determine whether magnesium ion could release the heart from vagal inhibition. In no case was this successful. At times the maximal inhibition produced by ten second stimulation of the vagus was measured every thirty seconds, and at other times the vagus was stimulated at a frequency sufficiently slow to prevent vagal escape. At no time was acceleration observed following an injection of magnesium chloride. With larger doses further slowing of the heart occurred. Possibly release from vagal tone could be shown if it were not for the direct slowing effect of magnesium ion on the sinus.

E. EFFECT OF MAGNESIUM ION ON THE BLOOD PRESSURE

Incidental to the experiments reported above a number of observations were made on the blood pressure. Regularly when magnesium chloride was injected intravenously a sharp fall in the blood pressure occurred. In no case was a rise observed. The pressure returned to normal or near normal in three to five minutes. With successive injections the base line of the blood pressure gradually fell until the animal died. However, frequently when the injection was made into the common carotid artery a sharp but transient elevation in the blood pres-

3. *Effect on synaptic transmission.* In experiments on three cats inhibition of ganglionic transmission was recorded with the cathode ray oscillograph. The stimulating electrodes were placed on the communicating branch from the first or second intercostal nerves for preganglionic stimulation and on the distal end

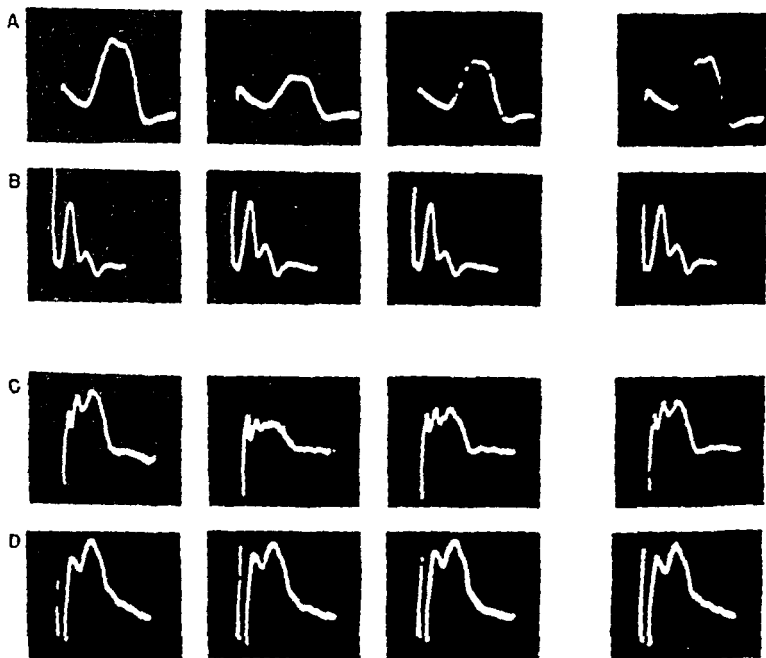


FIG. 4. ACTION POTENTIALS IN POSTGANGLIONIC FIBERS OF STELLATE AND INFERIOR MESENTERIC GANGLIA

Cat, male, 2.4 kg. in A and B; Cat, male, 5.2 kg. in C and D. Dial anesthesia. In each case 100 mgm. $MgCl_2$ was given intravenously during maximal stimulation of the appropriate nerve.

A. During stimulation of preganglionic fibers of the stellate ganglion. First control, second 15 seconds after injection; third 60 seconds after injection, and fourth 150 seconds after injection.

B. During stimulation of postganglionic fibers of the stellate ganglion. First control, second 15 seconds after injection, third 60 seconds after injection, and fourth 140 seconds after injection.

C. During stimulation of preganglionic fibers of the inferior mesenteric ganglion. First control, second 15 seconds after injection, third 60 seconds after injection, and fourth 180 seconds after injection.

D. During stimulation of hypogastric nerve. First control, second 15 seconds after injection, third 60 seconds after injection, and fourth 195 seconds after injection.

of the ganglion for postganglionic stimulation. The recording electrodes were applied to the cardiac nerve severed where it joined the vagus. Magnesium chloride injected intravenously in doses which caused cardiac slowing as described above regularly caused a decrease in the magnitude of the action potential when the stimulus was applied preganglionically (Fig. 4, A), but no change was

transmission of cardioaccelerator impulses at the stellate ganglion. It also has a direct decelerator action on the sino-atrial node. As with the contraction of the nictitating membrane so also with the heart rate, it has an inhibitory effect whether or not postganglionic stimulation is carried out. Since it caused no change in the amplitude of the action potentials in the accelerator nerve when the nerve was stimulated maximally distal to the ganglion, it can be assumed that the nerve impulses in the postganglionic fibers reached the heart. Since in the doses used, atrio-ventricular dissociation did not occur, the slowing was not due to changes in conduction within the heart. In dogs we have confirmed repeatedly the old observations of Matthews and Jackson (9) that the heart stopped by magnesium ion can be driven by suitably spaced electrical stimuli applied to the region of the sino-atrial node. Magnesium ion, then, slows the heart not only by diminishing cardioaccelerator tone, but also by diminishing the spontaneity of the sino-atrial node.

3. *Vasodepressor effects.* Since magnesium ion blocks impulse transmission through the superior cervical, the stellate, and the inferior mesenteric ganglia, it seems reasonable to assume that it blocks sympathetic ganglia generally. Hence, the blood pressure lowering effect of injected magnesium ion may be ascribed in part to its effect on these ganglia. Other sites of action might, however, be important. That the vaso-motor center is probably not particularly involved in the hypotensive effect of magnesium ion is indicated by the sharp rises in blood pressure after intracarotid injection. The site of this action is probably central since it occurred after evulsion of cranial nerves IX through XII.

The fall in blood pressure might also result from a direct effect on peripheral vessels or from a decrease in cardiac output. The observations here reported on the spinal cat and on a cat receiving a constant intravenous infusion of epinephrine do not differentiate between these possibilities, but do indicate that magnesium ion does not depend entirely on the central nervous system or the sympathetic nervous system in order to cause a lowering of blood pressure. Haury (10) found an increase in volume of spleen and kidney in the spinal animal after giving magnesium ion intravenously, and from his data concluded that it acts locally. Gotsev (11), however, using much larger doses, failed to find such changes unless the magnesium ion was injected intra-arterially, and concluded that the blood pressure fall was due to diminished output of the heart. More experimental work is required before the entire mechanism can be clarified.

CONCLUSIONS

1. Magnesium ion causes a short-lived blockade of transmission of nerve impulses through sympathetic ganglia. With comparable concentrations of magnesium ion conduction in pre- and postganglionic sympathetic nerves is little if any affected, but the threshold of electrical stimulation is raised.

2. Magnesium ion prevents the stimulating effect of acetylcholine and of potassium ion on the superior cervical ganglion.

3. Magnesium ion causes a slowing of the heart by blocking the cardioaccelerator ganglia, but in addition there is a direct inhibiting action on the spontaneity

sure was seen. This was sometimes followed by a secondary fall. Control injections of saline gave no rise. This was observed with and without evulsion of cranial nerves IX through XII.

Two experiments were done when sympathetic control of the blood pressure had been eliminated insofar as possible. In one of these the spinal cord and brain were destroyed. The blood pressure fell at once to about 60 mm. Hg. Intravenous injections of amounts of magnesium chloride which caused falls of blood pressure in the normotensive cat also caused falls in this animal. Another anesthetized cat (with intact nervous system) was given a slow intravenous drip of epinephrine 1/200,000 until the blood pressure stabilized at a level 88 mm. of mercury above the basal level. At this point graded doses of tetraethyl ammonium chloride led to rises of blood pressure instead of the usual fall, indicating that sympathetic tone had been eliminated. Injections of magnesium ion caused sharp falls in the blood pressure. These observations suggest that the blood pressure-lowering effect of magnesium ion is not entirely dependent on the presence of sympathetic vasoconstrictor tone.

DISCUSSION

1. *Blockade of sympathetic ganglia.* If magnesium ion is injected in proper amount during stimulation of the cervical sympathetic nerve a brief relaxation of the nictitating membrane is observed. The principal site of this activity has been localized at the superior cervical ganglion by showing that the nerve impulse is conducted undiminished in the pre- and postganglionic fibers when a quantity of magnesium ion is injected which blocks the ganglionic potential and transmission through the ganglion. This relatively brief blocking effect of magnesium cannot be attributed to changes in blood supply of the ganglion produced by the actions of magnesium, since ganglionic transmission is unaffected by decrease of blood supply short of several minutes of complete ischemia (8). Magnesium ion prevents the stimulating effect of potassium ion and of acetylcholine on the superior cervical ganglion. These effects also are exerted on the ganglion itself and not on the postganglionic fibers since they disappear when the ganglion is crushed. Thus magnesium ion renders the acetylcholine and potassium-sensitive portions of the ganglion relatively inexcitable to these agents. In this respect it differs from both curare and tetraethylammonium ion since both these drugs do not block the response of sympathetic ganglia to potassium but do block the response to acetylcholine. The initial stimulating effect on the ganglion which may be elicited by curare was never observed. In this respect the magnesium ion behaves more like tetraethylammonium ion.

Sympathetic ganglionic blockade was also shown in the stellate ganglion and in the inferior mesenteric ganglion. These were not well adapted for the study of acetylcholine and potassium ion effects, but otherwise the findings were consistent with those at the superior cervical ganglion in showing interference by magnesium ion with impulse transmission. The data presented are insufficient to define the mechanism by which this blockade takes place.

2. *Cardiodecelerator effect.* Magnesium ion slows the heart by blocking the

transmission of cardioaccelerator impulses at the stellate ganglion. It also has a direct decelerator action on the sino-atrial node. As with the contraction of the nictitating membrane so also with the heart rate, it has an inhibitory effect whether or not postganglionic stimulation is carried out. Since it caused no change in the amplitude of the action potentials in the accelerator nerve when the nerve was stimulated maximally distal to the ganglion, it can be assumed that the nerve impulses in the postganglionic fibers reached the heart. Since in the doses used, atrio-ventricular dissociation did not occur, the slowing was not due to changes in conduction within the heart. In dogs we have confirmed repeatedly the old observations of Matthews and Jackson (9) that the heart stopped by magnesium ion can be driven by suitably spaced electrical stimuli applied to the region of the sino-atrial node. Magnesium ion, then, slows the heart not only by diminishing cardioaccelerator tone, but also by diminishing the spontaneity of the sino-atrial node.

3. *Vasodepressor effects.* Since magnesium ion blocks impulse transmission through the superior cervical, the stellate, and the inferior mesenteric ganglia, it seems reasonable to assume that it blocks sympathetic ganglia generally. Hence, the blood-pressure lowering effect of injected magnesium ion may be ascribed in part to its effect on these ganglia. Other sites of action might, however, be important. That the vaso-motor center is probably not particularly involved in the hypotensive effect of magnesium ion is indicated by the sharp rises in blood pressure after intracarotid injection. The site of this action is probably central since it occurred after evulsion of cranial nerves IX through XII.

The fall in blood pressure might also result from a direct effect on peripheral vessels or from a decrease in cardiac output. The observations here reported on the spinal cat and on a cat receiving a constant intravenous infusion of epinephrine do not differentiate between these possibilities, but do indicate that magnesium ion does not depend entirely on the central nervous system or the sympathetic nervous system in order to cause a lowering of blood pressure. Haury (10) found an increase in volume of spleen and kidney in the spinal animal after giving magnesium ion intravenously, and from his data concluded that it acts locally. Gotsev (11), however, using much larger doses, failed to find such changes unless the magnesium ion was injected intra-arterially, and concluded that the blood pressure fall was due to diminished output of the heart. More experimental work is required before the entire mechanism can be clarified.

CONCLUSIONS

1. Magnesium ion causes a short-lived blockade of transmission of nerve impulses through sympathetic ganglia. With comparable concentrations of magnesium ion conduction in pre- and postganglionic sympathetic nerves is little if any affected, but the threshold of electrical stimulation is raised.
2. Magnesium ion prevents the stimulating effect of acetylcholine and of potassium ion on the superior cervical ganglion.
3. Magnesium ion causes a slowing of the heart by blocking the cardioaccelerator ganglia, but in addition there is a direct inhibiting action on the spontaneity

of the heart. Similarly, it causes an inhibition of the nictitating membrane contracted by stimulation of the cervical sympathetic nerve by blocking the superior cervical ganglion, but in addition there is a direct inhibiting action on the nictitating membrane.

4. The blood-pressure lowering action of magnesium is complex. It is probably not attributable to a central influence. Autonomic blockade, diminished cardiac output, and dilatation of vascular beds may be responsible, but the relative importance of each is not clear.

ACKNOWLEDGEMENT. The author wishes to express his gratitude to Dr. George H. Acheson for his help and advice during this work.

REFERENCES

1. JOLYET, F., AND CAHOURS: *Arch. de Physiol. Norm. et Path.*, 2: 113, 1869.
2. CLOETTA, M., FISCHER, H., AND VAN DER LOEFF, M. R.: *Arch. f. exper. Path. u. Pharmacol.*, 200: 6, 1942.
3. LANGLEY, J. N.: *J. Physiol.*, 52: 247, 1918.
4. ACHESON, G. H., AND MOE, G. K.: *THIS JOURNAL*, 87: 220, 1946.
5. ACHESON, G. H., AND PEREIRA, S. A.: *THIS JOURNAL*, 87: 273, 1946.
6. BROWN, G. L., AND FELDBERG, W.: *J. Physiol.*, 86: 10-11P, 1936.
7. ROSENBLUETH, A., AND CANNON, W. B.: *Am. J. Physiol.*, 125: 276, 1939.
8. BARGETON, D.: *Am. J. Physiol.*, 121: 261, 1938.
9. MATTHEWS, S. A., AND JACKSON, D. E.: *Am. J. Physiol.*, 19: 5, 1907.
10. HAURY, V. G.: *THIS JOURNAL*, 65: 453, 1939.
11. GOTSEV, T.: *Arch. f. exper. Path. u. Pharmacol.*, 201: 322, 1943.

COMPARATIVE PROPERTIES OF SIX PHENETHYLAMINES, WITH OBSERVATIONS ON THE NATURE OF TACHYPHYLAXIS¹

CLAUDE V. WINDER, MONA M. ANDERSON, AND HERVEY C. PARKE

Research Laboratories, Parke, Davis & Co., Detroit 32, Michigan

Received for publication December 19, 1947

Aralkyl and alkyl vasoconstrictor amines (1) are in use as volatile bases incorporated into inhalers for nasal decongestion. Not only are the relative merits of the two types an open question, but even within the first type to come into use, the aralkyl amines, there seems insufficient systematic basis for selection. We have concerned ourselves with the latter.

Consideration of volatility together with known structural requirements for reasonable pressor potency—which brackets vasoconstrictor potency—emphasize six compounds of the aralkyl class. They arrange themselves into the factorial design of table 1.

Further modification of the phenethylamine type is discouraging. Ring substitution or chain hydroxylation would probably cause inadequate volatility.

The two-carbon phenyl-to-nitrogen linkage is optimal for pressor action in the presence of a variety of substitutions (*e.g.*, (1-8)), including the aralkyl 2-imidazoline series (9). Either aliphatic substitutions heavier than methyl at the α -position of the chain (*e.g.*, (5, 6, 8, 10-14)), or carboxylation (3, 15) sharply diminishes or abolishes pressor action, except that dimethylation may not markedly reduce potency (16, 17). Heavier-than-methyl alkylation at the β -carbon may also be non-optimal (16), except that data on β -dimethyl substitution were not found. Methyl or heavier alkylation of both α - and β -carbons reduced potency (16). Secondary amines higher than the N-methyl (1, 6, 8, 11, 14, 16, 18), and tertiary amines (1, 6, 8, 10, 11, 17, 19, 20) have little or no potency. Quaternary amines are well known to assume different qualities of action. Conversion of the β -carbon to a ketone function apparently reduces pressor potency (1, 15, 21) and an ether function between ring and chain abolished it (3). Acetylation of the amine N, or replacement by guanidine, weakens potency (5).

Consequently, the convenient factorial design into which the six residual compounds arrange themselves encouraged assembling parallel data on some of their properties. Where asymmetric carbon atoms occur the racemic forms were used (III to VI). *L*-Ephedrine was included for comparison. Incidental to the original aims, observations arose concerning the nature of tachyphylaxis.

METHODS. Phenobarbitalized, atropinized dogs were used. Arterial pressure was recorded at the carotid artery for pressor potency assay alone; at the femoral artery when nasal decongestion and pressor potency or tachyphylaxis were studied simultaneously. In the latter case the vagosympathetic nerve trunks were severed to isolate the nasal mucosa from central sympathetic influences. Atropinization was depended upon to isolate the mucosa adequately from parasympathetic influences.

¹ Preliminary reports, Fed. Proc. 7(1), 1948.

The nasal-paranasal bony plethysmograph was closed off by Jackson's technic (22) slightly modified. Mucosal volume changes were recorded quantitatively with a miniature spirometer consisting of a small, light-weight, tin ointment cylinder, suspended from a lever designed to maintain counterpoise. The seal consisted of water containing a detergent for reduction of surface tension. Vertical recording distances were linearly related to volume displacements and were calibrated in connection with the nasal system.

Because of tachyphylaxis and influences of the amines on epinephrine responses, potency and duration assays were done in terms of a single injection of experimental amine per animal, following a descending dose scale (*cf.* Chen (23)) of epinephrine hydrochloride prepared from standard base. In the study of tachyphylaxis repeated doses were administered at intervals decreasing by 10-minute steps from 1 hr. to 10 min., the latter repeated several times. All injections were made quickly by syringe and needle into an exposed femoral vein, in normal saline, 0.1 cc./kgm. Doses and numbers of animals are shown in Table 2.

TABLE I

Six volatile phenethylamines arranged according to N-methylation and chain-carbon methylation

N-METHYLATION	CHAIN-CARBON METHYLATION		
	None	α -	β -
Absent	I β -Phenylethyl amine B.P., 194-196 M.P., 215-217	III β -Phenylisopropyl amine (Amphetamine) B.P., 203 M.P., 144-147	V β -Phenyl-n-propyl amine B.P. 104/21 mm. M.P., 138-140
Present	II β -Phenylethyl methyl amine B.P., 85/12 mm. M.P., 156-157	IV β -Phenylisopropyl methyl amine (Desoxyephedrine) B.P., 94-96/18 mm. M.P., 134-135	VI β -Phenyl-n-propyl methyl amine (Vonedrine) B.P., 100/14 mm. M.P., 145-146

Note:

B.P. is boiling point of base in °C; M.P. is melting point of hydrochloride.

I, II and IV were prepared in these laboratories.

III was obtained from Commercial Solvents Corporation.

V and VI were obtained from Chemical Specialties Co., Holland, Mich.

RESULTS

I. COMPARATIVE PROPERTIES.

The pressor potency of an amine was defined relative to that of epinephrine, according to common practice. For an individual determination, by graphic interpolation along a plot of the preliminary epinephrine dose-response scale, a dose of epinephrine was estimated which would have given a peak rise in mean arterial pressure equal to that given by the experimental amine. The potency of the amine relative to epinephrine was taken as the reciprocal ratio of the respective equipressor doses. For comparisons on a molecular basis, these ratios were adjusted according to the molecular weight of the experimental hydrochloride relative to that of epinephrine.

TABLE 2

Comparison of some properties of six volatile phenethylamine-type agents and *l*-ephedrine

AGENT	RELATIVE MOLECULAR PRESSOR POTENCY (EPINEPHRINE = 1) AT 46-178,† 250, AND 500 µGM./ KGM., 12-16 ANIMALS PER AMINE	RELATIVE DURATION (EPINEPHRINE = 1) AT 250 AND 500 µGM./KGM., 8-12 ANIMALS PER AMINE	INTRAVENOUS NASAL DECON- GESTANT EF- FICIENCY (DECONG. POTENCY/ PRESSOR POTENCY) (EPINEPHRINE = 1) AT 46- 178† µGM./KGM., 4 ANIMALS PER AMINE	TACHY- PHYLAXIS AT 174-571† µGM./KGM., 2-4 ANI- MALS PER AMINE
<i>l</i> -Ephedrine $C_6H_5 \cdot CH(OH) \cdot$ $CH(CH_3) \cdot NH(CH_3)$	0.0055 (0.0039-0.0077)	5.6 (4.6-6.9)	1.1 (0.15-7.7)	+++
I. β -Phenylethyl amine $C_6H_5 \cdot CH_2 \cdot CH_2 \cdot NH_2$	0.0066 (0.0044-0.0099)	1.5 (1.3-1.8)	0.28 (0.15-0.52)	±
II. β -Phenylethyl methyl amine $C_6H_5 \cdot CH_2 \cdot CH_2 \cdot$ $NH(CH_3)$	0.0057 (0.0041-0.0080)	1.2 (1.0-1.5)	0.26 (0.10-0.69)	±
III. (<i>d,l</i>) β -Phenylisopropyl amine $C_6H_5 \cdot CH_2 \cdot CH(CH_3) \cdot$ NH_2	0.0042 (0.0029-0.0061)	10.5 (6.1-18.0)	3.0 (0.66-13.0)	++++
IV. (<i>d,l</i>) β -Phenylisopropyl methyl amine $C_6H_5 \cdot CH_2 \cdot CH(CH_3) \cdot$ $NH(CH_3)$	0.0061 (0.0046-0.0081)	8.2 (5.9-11.4)	0.67 (0.33-1.3)	++++
V. (<i>d,l</i>) β -Phenyl- <i>n</i> -propyl amine $C_6H_5 \cdot CH(CH_3) \cdot CH_2 \cdot$ NH_2	0.0034 (0.0025-0.0048)	3.2 (2.7-3.8)	0.91 (0.44-1.9)	+
VI. (<i>d,l</i>) β -Phenyl- <i>n</i> -propyl methyl amine $C_6H_5 \cdot CH(CH_3) \cdot CH_2 \cdot$ $NH(CH_3)$	0.0024 (0.0017-0.0034)	2.9 (2.2-3.8)	0.52 (0.23-1.2)	+

All compounds were studied as hydrochlorides.

Figures in parentheses are 95% confidence limits computed in terms of experienced variance and Student's "t".

The three types of relative values are transformed to their logarithms for statistical summarization and analysis.

* Values within an individual bracket do not differ significantly; see text.

† Doses of respective amines estimated from the 250 and 500 µgm. dose levels per kgm. to be equipressor with 0.19 µgm. of epinephrine per kgm. Preliminary experiments had shown this level of epinephrine to be fairly well centered in the nasal decongestant effect range.

‡ Larger, approximately equipressor doses of respective amines.

There were strong suggestions that the non-chain-methylated (ethyl) amines (I and II) increased in potency relative to epinephrine with increasing dose level, whereas the α -methylated (isopropyl) amines (III and IV) decreased (cf. (23,

24)); but these results (regressions of log potency ratio on log dose) failed to reach statistical significance (5% level). Therefore, it seemed most conservative to summarize the potency of each amine as a (geometric) mean of potency ratios by all three dose levels used, with error expressed as of a single (non-regressive) sample but with dose range stated (table 2).

From analysis (25, 26) of the data according to chain- and nitrogen-methylation, only the former influenced potency with statistical significance ($F = 12.27$,

TABLE 3

Pressor ratios on a weight basis (hydrochlorides), compared with previously reported values (various methods and salt-forms)

COMPOUND	PRESENT VALUES (95% limits)	GEOM. MEAN OF REPORTED VALUES (Range)	REFERENCE
<i>l</i> -Ephedrine	0.0050 (0.0036-0.0070)	0.0035* (0.0016-0.0068)	(16, 23, 28) to (32)
I. β -Phenylethyl amine	0.0077 (0.0051-0.0116)	0.0107* (0.0055-0.0240)	(5, 7, 28, 33)
II. β -Phenylethyl methyl amine	0.0059 (0.0042-0.0082)	($\frac{1}{2}$ to = I)	(1, 5)
III. β -Phenylisopropyl amine	0.0043 (0.0029-0.0063)	0.0033† (0.0016-0.0071)	(5, 6, 28, 32) to (36)
IV. β -Phenylisopropyl methyl amine	0.0060 (0.0045-0.0080)	0.0016‡ (0.0007-0.0036)	(14, 35, 36)
V. β -Phenyl- <i>n</i> -propyl amine	0.0035 (0.0025-0.0049)	0.0015 (0.0010-0.0020)	(5, 16, 17, 35) to (37)
VI. β -Phenyl- <i>n</i> -propyl methyl amine	0.0024 (0.0017-0.0033)	0.0022 (0.0016-0.0040)	(16, 17, 36)

* In addition, the original work of Chen *et al.* (11, 38, 39) placed *l*-ephedrine less potent than I.

† Hartung and Munch (4) thought III equal to I, and Hauschild (8) placed it above *l*-ephedrine.

‡ Others have ranked IV as approximately equal to *l*-ephedrine (40), or equal (6) to or less (8) than III.

1

2 and 69 degrees of freedom). The β -methylated (*n*-propyl) amines (V and VI) were significantly less potent than the ethyl or isopropyl ($F = 22.75$ and 12.55 , 1 and 69 degrees of freedom). Difference between the ethyl and isopropyl failed to reach significance ($F = 1.51$, 1 and 69 degrees of freedom). There was a suggestion that N-methylation varied in its influence according to chain methylation, possibly decreasing potency of the ethyl and *n*-propyl, and increasing (or decreasing less) that of the isopropyl amines; but this, too, failed to reach significance (interaction; $F = 2.82$, 2 and 69 degrees of freedom).

The entire group of compounds was of a similar *order* of potency—so many thousandths of epinephrine on a molecular basis. Alles (27) and others have pointed out the fictitious accuracy of unqualified pressor values. Not only absolute and relative, but even rank values depend upon species, destruction of central nervous system *vs.* anesthesia, nature and depth of anesthesia, dose-level, and individual test animal. Differences in qualitative patterns of cardiovascular actions among the pressor amines and between them and epinephrine are implied. Table 3 will suffice to relate our results with values located in the literature. A study of the table emphasizes the need for definition of experimental conditions and statistical evaluation of data.

Duration of pressor effect was measured in terms of time from beginning of pressure rise to the point at which half-recovery had occurred—a sort of half-life of effect chosen to avoid the vagueness, and in the case of durable actions the great delay entailed by 'complete' recovery. Such a measure was also expected to be less dependent upon dose used. The ratio of a half-life to that of the (interpolated) epinephrine response of equivalent height was called the *relative duration*. It was necessary to compute these duration ratios from data at the two higher dose levels only, because at the low (adjusted) doses the half-life of the equipressor epinephrine response frequently ended on the descending limb of the well-known initial 'spike,' a characteristic not representative of the experimental amines.

The results are summarized in table 2. From analysis of the relative durations according to (a) dose, (b) N-methylation, and (c) chain-methylation, as in the case of potencies, only influences of chain-methylation were statistically significant ($F = 97.6$, 2 and 33 degrees of freedom). The isopropyl amines were most durable, the *n*-propyl were intermediate, and the ethyl least. Differences between the ethyl and isopropyl, ethyl and *n*-propyl, and isopropyl and *n*-propyl were all significant ($F = 194.0$, 36.44, and 62.27, 1 and 33 degrees of freedom). There was a consistent suggestion, failing to reach statistical significance, that N-methylation reduced duration ($F = 1.74$, 1 and 33 degrees of freedom).

There was no apparent relationship between potency and duration of action, although metabolic disposal must have a minor hidden influence on manifest potency. The more potent amines, the ethyl and isopropyl, were respectively least and most durable. The less potent *n*-propyl amines were intermediate in duration.

Intravenous nasal-decongestant potency was determined in a manner analogous to that of pressor potency except that the experimental response was matched against the scale of epinephrine decongestion, recorded simultaneously with pressor responses (fig. 1). The ratio of relative decongestant potency to relative pressor potency determined simultaneously in a given animal was considered a better-controlled quantity than the decongestant value as such. This amounts to *relative efficiency* of the amine as a decongestant, referred to its over-all pressor action, with epinephrine's efficiency set at unity (table 2).

Both chain-methylation and N-methylation significantly influenced decongestant efficiencies ($F = 15.83$, 2 and 18 degrees of freedom; and $F = 8.75$, 1 and

18 degrees of freedom). The least efficient were the ethyl amines (I and II). The *n*-propyl compounds ($F = 10.14$, 1 and 18 d.f.) and the isopropyl ($F = 31.46$, 1 and 18 d.f.) were considerably more efficient, the latter significantly more so than the former ($F = 5.88$, 1 and 18 d.f.). In general among the 6 compounds, the secondary amines were less efficient than the primary, with a suggestion that the inferiority resulting from *N*-methylation varied in amount according to chain-methylation (interaction; $F = 2.88$, 2 and 18 d.f.).

It is understood that inferences regarding relative mucosal *vasoconstriction* are subject to the reservation that central venous pressure was not controlled. If one cares to assume that relative differences in venous pressure at peak effect

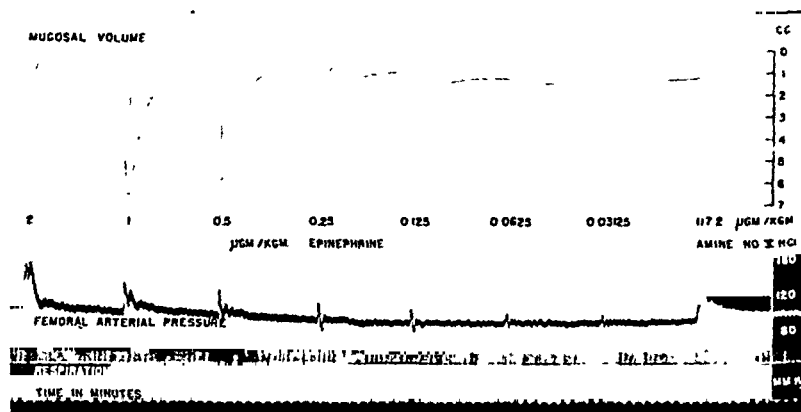


FIG. 1. EXPERIMENT ILLUSTRATING SIMULTANEOUS DETERMINATION OF PRESSOR AND NASAL DECONGESTANT POTENCIES

Dog, male, 14.5 kgm., anesthetized with sodium phenobarbital and atropinized. Vago-sympathetic nerves cut. Several 2 $\mu\text{gm./kgm.}$ injections of epinephrine (as HCl) precede the section of record shown, for establishing uniformity of response. Responses are subsequently plotted against logarithm of dose and epinephrine equivalents of the test injection determined by interpolation.

were unimportant in determining relative mucosal shrinkage among the amines, then the *decongestion* efficiency may be considered as presumptive evidence of intravenous nasal mucosal *vasoconstrictor* efficiency. It is interesting that the amine which is indicated to be most efficient—the isopropyl primary amine (III, *d,l*-amphetamine)—has had a long-established use as a volatile nasal decongestant. Conversely, the two ethyl amines (I and II) were significantly less efficient than epinephrine, and although potent *general* pressor agents and perhaps the 'oldest' pharmacologically of the group, they are not used clinically.

The data suggest that the commercially unexploited primary amine (V) of the *n*-propyl pair may be a more efficient decongestant than the secondary amine.

Tachyphylaxis was very little more evident with the parent amine (I) and its *N*-methyl derivative (II) than with epinephrine (1.37 $\mu\text{gm./kgm.}$) in either the rise in blood pressure or nasal decongestion. Effects tended rather to increase

than decrease during a large part of the experimental period. There was a variable tendency toward diminishing effect during the last few short-interval injections. The conflicting reports on tachyphylaxis (and duration of action) of phenethylamine found in the literature (5, 7, 27, 28, 33) may perhaps be explained in terms of dose and time interval (*vide infra*).

The two *n*-propyl compounds (V and VI) exhibited evidence of tachyphylaxis at the 5th to 6th injection, at the 20-min. or first 10-min. interval, or about 3½ hours after the first injection. That is, the experimental design was just adequate for demonstration of definite tachyphylaxis in these two. No difference between the primary and secondary amine was apparent.

The two isopropyl compounds (III and IV) presented the third pair of similar behaviors. As expected from well-known properties of both of them, tachyphylaxis was markedly apparent even at the second injection, though separated by a 1-hr. interval from the first. Succeeding injections brought responses down at a decreasing rate nearly to zero at the 10th injection, 4 hrs. from the first. Initial falls in pressure preceded the rise from the second to fourth injection onward, and only the fall was notably present at the end.

L-Ephedrine was less precipitously tachyphylactic as regards its pressor action than the non- β -hydroxylated isopropylamines (III and IV). There was still a 20-23 mm. Hg pressor action at the 10th injection.

There was a striking parallelism of tachyphylaxis in pressor and decongestant responses, as illustrated in figure 2. It is now known that tachyphylaxis to ephedrine and related isopropylamines is not limited to the heart (23, 41, 42, 43), but is demonstrable on the composite vascular bed (44, 45), various vascular beds (44, 46-49), spleen (50), uterus (19, 47, 51, 52), respiratory control (34), intestinal musculature (34, 53, 54), bronchial musculature (39, 41), etc. Tachyphylaxis of ephedrine has previously been observed with nasal decongestion (55).

Several qualitative experiments showed that the tachyphylactogenic properties of ephedrine and of the two (non-hydroxylated) isopropyl amines crossed over to influence responses to the *n*-propyl amines as well as those to themselves, and *vice versa*; and also, that the two *n*-propyl amines were cross-tachyphylactic, in the much lesser degree of their self-tachyphylaxis. Crossed tachyphylaxis among pressor amines and also influences between pressor amines and less closely allied agents have been repeatedly described (15, 23, 40, 46, 51, 52, 54, 56), even for the (primary) depressor component of action which many exhibit (20).

Comment. If therapeutic considerations should demand a volatile decongestant of intermediate duration, intermediate tachyphylactic liability, low degree of higher nervous action (8, 16, 57-59), and freedom from liability of systemic action (relative facility of metabolic disposal) by mucosal absorption (8, 59-66), β -phenyl-*n*-propyl amine, with or without *N*-methylation, is of interest. As concerns potency, duration, tachyphylaxis, and intravenous nasal-decongestant efficiency relative to over-all pressor action, the primary amine seems at least as deserving of attention as the secondary. Topical efficiency and local toleration would need to be examined directly. Preliminary data suggest that it may be the least volatile of the six (under simulated practical inhaler conditions),

but not greatly inferior, and this factor may be amenable to pharmaceutical adjustment.

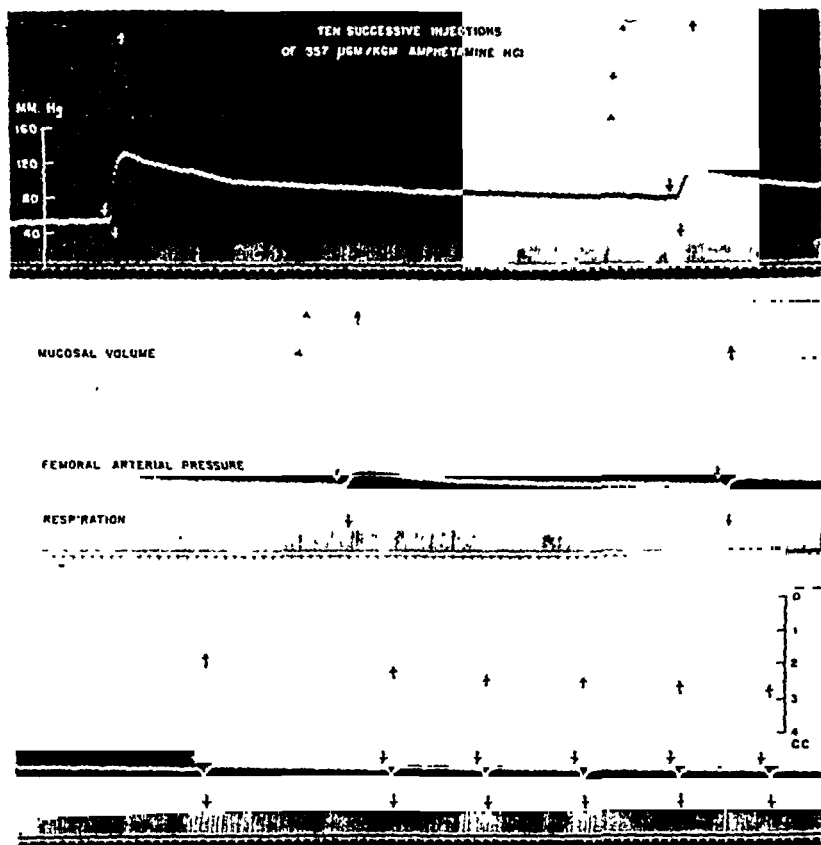


FIG. 2. TACHYPHYLACTIC RESPONSES TO *d,l*-AMPHETAMINE

Dog, male, 13.4 kgm., anesthetized with sodium phenobarbital and atropinized. Vago-sympathetic nerves cut. Continuous tracing. Dashed arrow in plethysmogram indicates adjustment of plethysmograph. Note parallelism of tachyphylaxis in nasal mucosa and in over-all blood pressure, and maintenance of nasal decongestion.

II. ON THE NATURE OF TACHYPHYLAXIS AND RELATED PHENOMENA.

It has been mildly polemic (50, 67) whether diminishing effect of repeated doses of an agent on a function such as arterial pressure should be termed tachyphylaxis when persisting effects of preceding doses are apparent. But such persistence of change in an agent, or factor of general functional level, must commonly be masked by compensatory and/or deteriorative changes in other factors, hence rendered *non*-apparent. This is illustrated in figure 2 where the nasal plethymo-

gram indicates a definitely persistent drug-induced vasoconstriction, uncompensated in the neurally-isolated mucosa, but, as a blood pressure factor, well-compensated for the organism as a whole and rendered non-apparent in blood pressure (excepting the first procedure). Thus, such polemics lose much fundamental meaning. Indeed, a current hypothesis that tachyphylaxis of pressor amines (and certain other agents) basically reflects persistent dynamic blockade of biological 'receptor surfaces' (51, 52, 68, 69) would inherently require persistence of the agent, intact or modified. Results such as figure 2 indicate that the agent may persist intact to an important degree at least.

The blockade hypothesis fits the co-existence of metabolic refractoriness (66) with more or less manifest persistence of functional effect, duration of action, and tachyphylaxis in the isopropylamines. That the blockade may be in the nature of a dynamic equilibrium with the receptor material (69), and perhaps largely in the form of unaltered agent, is attested to by eventual recovery of large proportions or nearly all of the strongly tachyphylactic isopropylamines in the urine (66, 70-73).

It seems reasonable to suppose that the proportion of a population of receptor points dynamically occupied by the agent would be in the nature of a statistical cumulation according to the agent's concentration in the cellular environment; that is, that there would be a cumulative probability of receptor occupation along a dose scale. In view of the nearly normal distribution of most biological *quantal* effects on the logarithmic scale of agent dose, it seems reasonable that the statistical occupation of *receptor points* might also be normalized on the logarithmic scale of dose. Because of the manner of cumulation of pyramided statistical samples (in this case, successive, converging, physico-chemical and anatomical levels (74, 75)), the same fundamental form of probability curve might reasonably be expected to reflect itself in the relation between measured end-effect, and logarithmic dose (74). This principle has, indeed, been found to hold in several sets of *quantitative* data (74, 75).²

To test such a manifold hypothesis the effect, as per cent of maximal effect, is plotted on a probability scale (or per cent converted to probits (Bliss) and plotted linearly) against the logarithm of dose/kgm. To include in the test the supposition that progressive tachyphylaxis reflects additive occupation of receptor points by the agent, 'effect' becomes *additive* effect (addition of effects of all preceding injections through the current effect), plotted as per cent of maximum effect, and 'log dose/kgm.' becomes the logarithm of *additive* dose/kgm. In the simplest case, excretion or other disposal of the agent may be regarded as negligible during the course of the acute experiment. However, unless the final injection of a tachyphylactic series results in zero effect, the maximal effect has not been directly determined and must be arrived at by addition of an estimated remainder to the sum of all actual recordings.

The decongestion plots of figure 3 are visual fits attained by trial and error settlement on such remainders. The remainders amounted to 2.7 to 9.7% of the resulting estimated maximal decongestion, which seemed reasonably in line

² See addendum.

with the small effects recorded from last injections. The figure includes all experiments wherein tachyphylaxis was nearly enough complete to enable such estimates; i.e., the cases of the isopropylamines except one ephedrine experiment. Blood pressure data fitted the hypothesis with slightly more deviation (from linearity), perhaps partly because of the vigor and complexity of compensatory mechanisms (74) and resulting distortion of blood-pressure response as a scale of measurement of receptor occupation.

The linear fits are amazingly satisfactory. It seems incredible that the entire set of assumptions should have added up to so little deviation from hypothesis in all experiments. But perhaps the most incredible assumption is neglect of disposal of the agent during the course of the experiment. It can, indeed, be shown that *with the experimental schedule used*, partial loss of the cumulating agent between injections would result in some concavity of the plots, particularly at the upper part, caused by recording overlapping segments of the effect range. However, it can then be shown that when assumed disposal rates are not in excess of about 40% per hour, somewhat enlarging the estimate of maximal effect brings the plots to near linearity with no more of the slight, sigmoid deviation than exists in any of the experiments (fig. 3). Thus, the small rate of disposal of these metabolically stable amines expected in the anesthetized dog is not critical to the test of linearity, hence of the other assumptions. Since after selection of the maximal effect which gives the best fit, the line is somewhat lower and less steep than would be the ideal case with no loss of agent, dose-effect parameter estimates cannot be made safely.

The experimental data strikingly satisfy the basic hypotheses: (a) that the pharmacological effect is graded in terms of cumulative probability of receptor occupation, according to the logarithm of agent concentration; and (b) that tachyphylactic responses merely reflect successive stages of cumulative probability of receptor occupation with successive (diminishing logarithmic) increments of agent. This becomes more clear with the aid of figure 4, where experiment C of figure 3 is plotted with the ordinate scale reconverted to the more familiar units of measurement of effect, and where successive response heights are indicated on the right.

The question arises as to what influences the hypothesis would predict from (a) dose or initial-effect level and (b) degree of metabolic disposal of the agent between injections. The experiment of figure 2 (C of figure 3 and the corresponding extrapolated curve of fig. 4) lends itself to such considerations. It is believed to represent an actual case of negligible disposal of agent. Curve C of figure 3 is seen to show little or no evidence of the sigmoid deviation which disposal would entail (*supra*); and more critically, the estimated maximal blood pressure rise (145 mm. Hg.) giving the best fit for the blood pressure plot apparently was not exaggerated as would have been the case with overlapping of successive effects along the effect scale if significant disposal had occurred. Consequently, C of figure 3 and the corresponding curve of figure 4 are believed to represent closely the dose-effect relationship for amphetamine in this animal. Now, if one assumes various sizes of the dose to be repeated, and, for each dose,

various percentages disposal of the cumulated agent between injections, the manners of variation in successive responses corresponding to given dose-disposal combinations can be predicted quantitatively. This has been done in figure 5-A, where a major implication of the hypothesis at once reveals itself; *viz.*, that at low dose levels the opposite of tachyphylaxis—a *self-potential*—may occur on repetitive administration of an agent such as amphetamine. It is

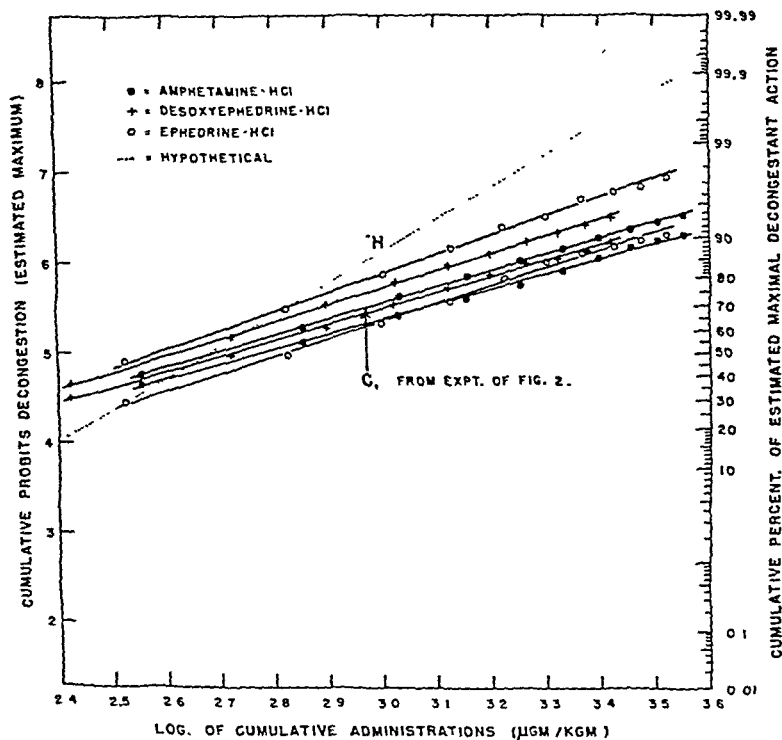


FIG. 3. CUMULATIVE TACHYPHYLACTIC RESPONSES EXPRESSED AS PERCENT OF AN ESTIMATED MAXIMAL RESPONSE, WHEN PLOTTED ON A PROBABILITY SCALE, PROGRESS IN LINEAR PROPORTION TO THE LOGARITHM OF CUMULATIVE DOSES

apparent, however, that the level of dose or initial-effect at which self-potential would be expected is so low, and the phenomenon itself so mild (even with zero disposal), that it would ordinarily be confounded with experimental error and only tachyphylaxis would be noteworthy for the drug.

Another question arises, as to the predicted influence of steepness of the curve relating receptor occupation with logarithmic dose; that is, compactness of probability range of agent-receptor engagement. The dotted curves (H) of figures 3 and 4 represent a hypothetical agent-receptor system with a doubly compact range, or doubled probit-effect-on-log-dose slope. Figure 5-B sets out the pre-

dictions concerning repetitive injections for initial-effect levels and percentages of disposal corresponding to those of figure 5-A. The hypothetical influences of increased dose-effect slope are striking. Self-potential is now large, and occurs at high enough effect levels that it would not be lost experimentally. It is particularly marked and persistent if one hits upon an optimal per cent disposal, which increases with dose-effect level. Tachyphylaxis, too, (at the higher

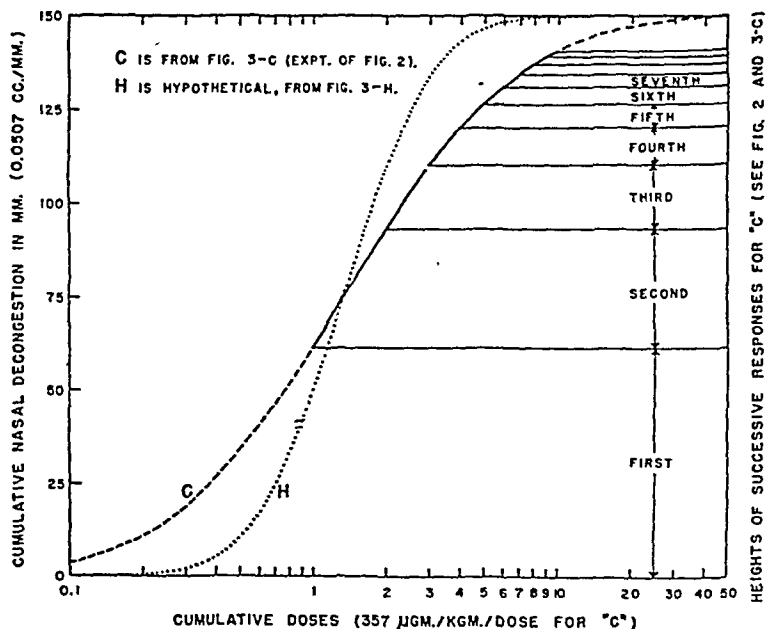


FIG. 4. "C" OF FIGURE 3 (FROM EXPERIMENT OF FIG. 2) REPLOTTED ON THE MORE FAMILIAR ORDINATE SCALE

Solid portion of curve is actual experimental range (Fig. 2 and 3); dashed portions are extrapolations according to normal probability. Successive responses represent successive segments of cumulative probability of receptor-substance engagement according to logarithm of cumulative dosage. Compensatory and/or deteriorative factors may more or less restore original functional level between administrations, as in arterial pressure of figure 2. There was evidence that disposal of agent between injections was negligible in this experiment (see text).

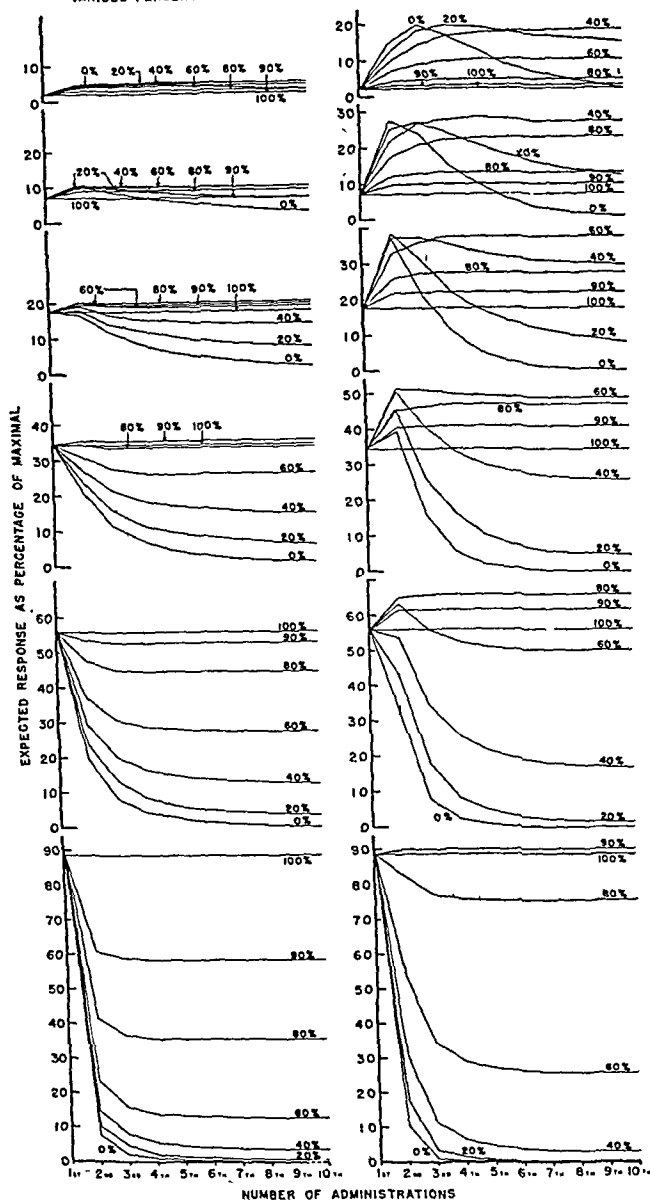
initial-effect levels and lower disposal rates) is more pronounced although more liable to be preceded by self-potential.

Such requirements of the hypothesis can be tested, and, in the testing, qualifying phenomena will undoubtedly emerge. The concept meets certain general tests present in these experiments and in the literature. It has been clear since Chen's work (76) that accentuation of tachyphylaxis depends importantly on size of dose and interval between injections. The latter is one factor determining the percentage disposal between injections (*cf.* fig. 5). Chen (76, 77) also ob-

A.-FROM "C" OF FIG. 3 AND 4.

B.-FROM "H" OF FIG. 3 AND 4.

VARIOUS PERCENTAGES OF DISPOSAL BETWEEN ADMINISTRATIONS



NUMBER OF ADMINISTRATIONS

FIG. 5

A.—Hypothetical series of responses to repeated administrations of uniform dose computed from experiment of figure 2 ("C" of Fig. 3 and 4) for various dose (or initial effect) levels and various degrees of disposal of agent between administrations.

B.—Same for a hypothetical agent-receptor system with more compact probability range of engagement ("H" of Fig. 3 and 4).

Note the kaleidoscopic manner in which amount of self-potential *vs.* amount of tachyphylaxis are expected to vary according to (a) compactness of probability range characteristic of the agent-receptor system, (b) dose, (c) disposal between administrations (rate of disposal and time between injections), and (d) number of injections.

served that small repeated doses (e.g., 1 mgm./dog) of ephedrine result in (summed or non-summed) approximately equal effects, following which a large dose gives a large effect (cf. first 2 or 3 initial-response levels of fig. 5-A). Size of dose, etc., might explain Chen's (23) early lack of evidence of tachyphylaxis from ephedrine on uterine strips. It is not surprising that at borderline doses of various isopropylamines the evidence for or against tachyphylaxis has appeared different to various workers (6, 40, 46, 55, 78-80). The outcome for a given drug would depend partly on the number of injections made (cf. fig. 5).

Another early observation on ephedrine (45) was that the difference between the first and second response was greater than differences among subsequent responses. This has been a common experience with isopropylamines, repeated in the present work. Inspection of figure 5-A predicts it for the moderate to large doses commonly used to demonstrate tachyphylaxis, but not for small doses. However, with steeper dose-effect relationships (e.g., fig. 5-B), it would not be true except for still larger doses, and then provided that no greater than a certain percentage disposal occurred between injections. The greatest evidence of disposal of agent between injections in the present isopropylamines occurred with ephedrine, in the form of the greater sigmoid deviation from linearity in figure 3 and seemingly exaggerated total effects (cf. also shorter average duration of effect, table 2). Even though this would result in a diminished apparent log dose-probit slope (*vide supra*), the ephedrine slope remains greater than for the non-hydroxylated isopropylamines (fig. 3). Greater true dose-effect steepness together with a perceptible rate of disposal might be expected (cf. fig. 5) to lead to less precipitous tachyphylaxis at a given initial-effect level, as was actually observed, and also to chances of less difference between first and second responses than between second and third, which actually occurred in all blood pressure series and one of the nasal decongestant series with ephedrine.

With a steep dose-effect relationship, repetition of a moderate dose could yield a doubly-flexed course of tachyphylaxis which may or may not be preceded by self-potential (fig. 5-B), depending on disposal between injections and actual dose. In the present experiments there was indeed evidence of self-potential for several injections with the two phenethyl amines (I and II) and the *n*-propyl amines (V and VI). Only after several injections and/or decreasing interval did the responses decrease, particularly in the latter two cases (V and VI). This suggests, back through the hypothesis, a steeper dose-effect relationship for I, II, V, and VI than for the isopropylamines, which recalls suggestive evidence of the same from the pressor assays. Many laboratories must have filed experiments in which a series of epinephrine responses at short intervals increased and then reached a plateau (cf. fig. 5). Also, diminishing responses with very large doses of epinephrine, particularly if closely spaced, are not unexpected either in practice or from present theory.

The literature contains many observations on augmentation or diminution of response to a pressor amine (or other agent) by administration of another pressor amine (or other agent). It is suggested that quantitative study of these effects from the view of the present concept will aid in determining whether there is a

statistical correlation between receptor-points of test agent and interfering agent (81), and otherwise add insight into modes of action. For example, moderate doses of certain isopropylamines enhance epinephrine responses while very large doses diminish or nearly prevent them (52, 54, 69), *suggesting* such correlation.

Reversal of effects would be regarded as selective influence on one of two or more receptor-point populations, as already proposed by others.

The present concept renders the phenomena of tachyphylaxis, cross-tachyphylaxis, self-potential, cross potential, and passive interference, perhaps to varying degrees, kaleidoscopic manifestations of varying patterns of receptor-point occupation according to concentrations, inherent average affinities, rates of disposal, time intervals, steepnesses of probits of receptor occupation according to relative dose, and the numbers of administrations.

The idea is non-committal as to whether the action (hence receptor population) is directly on the effector cell or indirectly, *e.g.*, on an enzyme whose inactivation would cause manifestation of latent action of a physiological agent (epinephrine (61, 68)), or both.

It seems that such thoughts will be of interest in such widely varying fields as those of the opiates and of antigen-antibody reactions. Development of 'tolerance' to opiates may be closely analogous to tachyphylaxis of pressor agents. The basis of physical addiction may be fundamentally similar to the mechanisms which tend to restore blood pressure to "normal" between tachyphylactogenic injections of a cumulating pressor agent; namely, appearance of compensatory and/or deteriorative functional factors, more or less completely keeping pace with and hence annulling the cumulating direct functional influences of the opiate. 'Withdrawal' phenomena may reflect more or less of the masking pattern left unopposed by more rapidly receding direct effects of the agent. Thus, whether withdrawal phenomena are definite, as with the opiate, or debatable, as with amphetamine (82), would depend importantly on definiteness or debatableness of disparity in recessions of direct and indirect (masking) patterns of effects. The quality and temporal course of withdrawal phenomena would depend importantly on the quality of direct and reaction (masking) patterns of effects. Even though the primary therapeutic action of two agents such as morphine and methadon may be very similar, and even though extensive tolerance may develop to that action in either case, the patterns and sites of direct side-effects and their reactions may be responsible for strikingly different withdrawal phenomena (83).

SUMMARY

1. The hydrochlorides of phenethylamine, its alpha- and beta-methylated derivatives, and the three corresponding N-methyl compounds were studied along with *l*-ephedrine in phenobarbitalized, atropinized dogs. Racemic forms were used where asymmetry occurred in the former six.

2. Average pressor potency (46–500 $\mu\text{gm./kgm.}$ dose level) on a molecular basis ranged within the same order of magnitude for the 7 compounds. The beta-methylated compounds were significantly less potent than the others.

3. Intravenous nasal-paranasal decongestant action (46–178 $\mu\text{gm./kgm.}$ level) was recorded volumetrically with vago-sympathetic nerves cut. Decongestant efficiency was defined as the ratio of decongestant potency to pressor potency as determined against respective epinephrine scales from a common set of injections—an efficiency index with epinephrine set at unity. The parent amine and its N-methyl derivative were least efficient (significantly less than epinephrine), the beta-methylated intermediately, and the alpha-methylated most. In general, the primary amines were more efficient than the secondary. Inferences concerning nasal *vasoconstrictor* efficiency can be made with reservations.

4. Durability of action was measured in terms of time from beginning of effect until recession to half-value. Its ratio to the same measure of the equipressor epinephrine response was termed the relative duration and was satisfactorily independent of dose level. Tachyphylaxis was studied systematically by repeated injections at decreasing time intervals of approximately pressor-equivalent doses (174–571 $\mu\text{gms./kgm.}$). The non-chain-methylated amines were least, the beta-methylated amines intermediately, and the alpha-methylated compounds most durable and tachyphylactic. *l*-Ephedrine was somewhat less durable and less precipitously tachyphylactic than the non-hydroxylated isopropylamines.

5. The tachyphylactic responses were found to fit with quantitative precision the hypothesis that they represent successive (more or less overlapping) stages of cumulative normal probability of receptor-point occupation by the agent, according to the logarithm of its cumulative concentration. Tachyphylaxis and self-potential seem to an important extent to be complementary phenomena, critically dependent upon the dose or initial probit-effect level, the steepness of the log. dose-probit effect curve (compactness of probability range of agent-receptor engagement), the rate of disposal of the agent, the time interval between injections, and the number of injections made. The over-all level of the function being cumulatively influenced by the agent may more or less completely return to the initial between injections, as in the case of the vigorously compensated cardiovascular system as a whole, or may not, as in the vascular bed of the neurally isolated nasal mucosa of the present experiments.

ADDENDUM

Among sets of data studied while considering the *statistical concept of gradation of 'quantitative' biological effect* (74) was that of Wells et al. (ref. 37 of (74)) on the depressor action of histamine and its antagonism by diphenhydramine hydrochloride. The log dose-probit fits were satisfactory. It is therefore interesting that Graham, Chen et al. (THIS JOURNAL, 92: 90, 1948) have now found the probit-regression applicable to quantitative actions of these same agents on isolated ileal strips. While Chen tentatively considered this to favor the 'all-or-none' theory of smooth muscle contraction, he agrees (personal communication) that the 'probit' concept of 'quantitative' response accommodates 'unitary' grada-

tion at levels beyond the initial physico-chemical level, so that a choice between 'all-or-none' and graded action of units at any such level is unnecessary (74).

REFERENCES

1. BARGER, G., AND DALE, H. H.: *J. Physiol.*, **41**: 19, 1910.
2. TAINTER, M. L.: *Quart. J. Pharm. & Pharmacol.*, **3**: 584, 1930.
3. HASAMA, B.: *Arch. f. exper. Path. u. Pharmacol.*, **153**: 161, 1930.
4. HARTUNG, W. H., AND MUNCH, J. C.: *J. Am. Chem. Soc.*, **53**: 1875, 1931.
5. TAINTER, M. L.: *Arch. internat. de pharmacodyn. et de therap.*, **46**: 192, 1933.
6. LARSEN, V.: *Skand. Arch. f. Physiol.*, **79**: 282, 1938.
7. GUNN, J. A., AND GURD, M. R.: *J. Physiol.*, **97**: 453, 1940.
8. HAUSCHILD, F.: *Arch. f. exper. Path. u. Pharmacol.*, **195**: 647, 1940.
9. HARTMANN, M., AND ISLER, H.: *Arch. f. exper. Path. u. Pharmacol.*, **192**: 141, 1939.
10. LOEWI, O., AND MEYER, H.: *Arch. f. exper. Path. u. Pharmacol.*, **63**: 213, 1906.
11. CHEN, K. K., WU, C.-K., AND HENRIKSEN, E.: *THIS JOURNAL*, **36**: 363, 1929.
12. HARTUNG, W. H., MUNCH, J. C., DECKERT, W. A., AND CROSSLEY, F.: *J. Am. Chem. Soc.*, **52**: 3317, 1930.
13. SCHAUHMANN, O.: *Arch. f. exper. Path. u. Pharmacol.*, **160**: 127, 1931.
14. NOVELLI, A. N., AND TAINTER, M. L.: *THIS JOURNAL*, **77**: 324, 1943.
15. TAINTER, M. L.: *Arch. internat. de pharmacodyn. et de therap.*, **41**: 365, 1931.
16. WARREN, M. R., MARSH, D. G., THOMPSON, C. R., SHELTON, R. S., AND BECKER, T. J.: *THIS JOURNAL*, **79**: 187, 1943.
17. ORTH, O. S., STUTZMAN, J. W., AND MEEK, W. J.: *THIS JOURNAL*, **81**: 197, 1944.
18. BARBOUR, H. G.: *THIS JOURNAL*, **8**: 126, 1916.
19. CURTIS, F. R.: *THIS JOURNAL*, **35**: 321, 1929.
20. KONZETT, H.: *Arch. f. exper. Path. u. Pharmacol.*, **197**: 41, 1941.
21. TAINTER, M. L.: *THIS JOURNAL*, **40**: 43, 1930.
22. JACKSON, D. E.: *Experimental Pharmacology and Materia Medica*, 2nd Ed., The C. V. Mosby Co., St. Louis, 1939.
23. CHEN, K. K.: *THIS JOURNAL*, **33**: 237, 1928.
24. HJORT, A. M., DE BEER, E. J., AND RANDALL, L. O.: *THIS JOURNAL*, **71**: 105, 1941.
25. FISHER, R. A.: *Statistical Methods for Research Workers*, 7th Ed., Oliver and Boyd, Edinburgh and London, 1938.
26. SNEDICOR, G. W.: *Statistical Methods*, 4th Ed., The Collegiate Press, Inc., Ames, 1946.
27. ALLES, G. A.: *Univ. California Publ. in Pharmacol.*, **1**: 129, 1938-1941.
28. CRISMON, C. A., AND TAINTER, M. L.: *THIS JOURNAL*, **66**: 146, 1939.
29. ROHRMANN, E., AND SHONLE, H. A.: *J. Am. Chem. Soc.*, **66**: 1516, 1944.
30. ROWE, L. W.: *J. Am. Pharm. Assoc.*, **16**: 912, 1927.
31. TAINTER, M. L.: *THIS JOURNAL*, **36**: 569, 1929.
32. ORTH, O. S., LEIGH, M. D., MELLISH, C. H., AND STUTZMAN, J. W.: *THIS JOURNAL*, **67**: 1, 1939.
33. ALLES, G. A.: *THIS JOURNAL*, **47**: 339, 1933.
34. DETRICK, L. E., MILLIKAN, R., MODERN, F. S., AND THIENES, C. H.: *THIS JOURNAL*, **60**: 56, 1937.
35. SWANSON, E. E., SCOTT, C. C., LEE, H. M., AND CHEN, K. K.: *THIS JOURNAL*, **79**: 329, 1943.
36. GRAHAM, B. E., CARTLAND, G. F., AND WOODRUFF, E. H.: *Ind. Eng. Chem.*, **37**: 149, 1945.
37. GRAHAM, B. E., CARTLAND, G. F.: *THIS JOURNAL*, **81**: 360, 1944.
38. CHEN, K. K.: *Arch. Int. Med.*, **39**: 404, 1927.
39. SWANSON, E. E.: *THIS JOURNAL*, **36**: 541, 1929.
40. HAUSCHILD, F.: *Klin. Wchnschr.*, **17**: 1257, 1938. *Arch. f. exper. Path. u. Pharmacol.*, **191**: 465, 1938.

41. CHEN, K. K., AND SCHMIDT, C. F.: *THIS JOURNAL*, 24: 339, 1924-1925.
42. HILDEBRANDT, F., AND MÜGGE, H.: *Klin. Wehnschr.*, 10: 291, 1931.
43. CRISMON, J. M., AND TAINTER, M. L.: *THIS JOURNAL*, 64: 190, 1938.
44. MÜGGE, H.: *Arch. f. exper. Path. u. Pharmacol.*, 165: 230, 1932.
45. TAINTER, M. L.: *Arch. internat. de pharmacodyn. et de therap.*, 42: 186, 1932.
46. PAK, C., AND READ, B. E.: *Chinese J. Physiol.*, 2: 435, 1928.
47. SCHAUMANN, O.: *Arch. f. exper. Path. u. Pharmacol.*, 138: 208, 1928.
48. PAK, C., AND KING, T.: *Chinese J. Physiol.*, 4: 141, 1930.
49. MORTON, M. C., AND TAINTER, M. L.: *J. Physiol.*, 98: 263, 1940.
50. RIECHERT, W., AND GULDIN, H.: *Arch. f. exper. Path. u. Pharmacol.*, 200: 244, 1942-1943.
51. CURTIS, F. R.: *THIS JOURNAL*, 35: 333, 1929.
52. MALORNY, G., AND ORZECOWSKI, G.: *Arch. f. exper. Path. u. Pharmacol.*, 196: 245, 253, 260; 1940.
53. SEIDENFELD, M. A., AND TAINTER, M. L.: *Proc. Soc. Exper. Biol. & Med.*, 28: 612, 1930-1931.
54. EICHLER, O.: *Arch. f. exper. Path. u. Pharmacol.*, 187: 429, 1937.
55. KING, T., AND PAK, C.: *Chinese J. Physiol.*, 3: 95, 1929.
56. NAGEL, A.: *Arch. f. exper. Path. u. Pharmacol.*, 110: 129, 1923.
57. TAINTER, M. L., WHITSELL, L. J., AND DILLE, J. M.: *THIS JOURNAL*, 67: 56, 1939.
58. SCHULTE, J. W., REIF, E. C., BACHER, J. A., JR., LAWRENCE, W. S., AND TAINTER, M. L.: *THIS JOURNAL*, 71: 62, 1941.
59. WARREN, M. R., AND WERNER, H. W.: *THIS JOURNAL*, 85: 119, 1945.
60. HAUSCHILD, F.: *Klin. Wehnschr.*, 20: 363, 1941.
61. BLASCHKO, H., RICHTER, D., AND SCHLOSSMANN, H.: *Biochem. J.*, 31: 2187, 1937.
62. BEYER, K. H.: *THIS JOURNAL*, 71: 151, 1941.
63. BEYER, K. H., AND LEE, W. V.: *THIS JOURNAL*, 74: 155, 1942.
64. BEYER, K. H.: *THIS JOURNAL*, 79: 85, 1943.
65. SNYDER, F. H., GOETZE, H., AND OBERST, F. W.: *THIS JOURNAL*, 86: 145, 1946.
66. BEYER, K. H.: *Physiol. Rev.*, 26: 169, 1946.
67. REIN, H.: *Arch. f. exper. Path. u. Pharmacol.*, 187: 617, 1937.
68. GADDUM, J. H., AND KWIATKOWSKI, H.: *J. Physiol.*, 94: 87, 1938-1939.
69. ORZECOWSKI, G.: *Arch. f. exper. Path. u. Pharmacol.*, 198: 27, 1941.
70. RICHTER, D.: *Bioch. J.*, 32: 1763, 1938.
71. JACOBSEN, E., AND GAD, I.: *Arch. f. exper. Path. u. Pharmacol.*, 196: 34, 280; 1940.
72. LARSEN, V.: *Arch. f. exper. Path. u. Pharmacol.*, 196: 43, 1940.
73. BEYER, K. H., AND SKINNER, J. T.: *THIS JOURNAL*, 68: 419, 1940.
74. WINDER, C. V.: *Yale J. Biol. & Med.*, 19: 289, 1947.
75. WINDER, C. V., AND THOMAS, R. W.: *THIS JOURNAL*, 91: 1, 1947.
76. CHEN, K. K.: *THIS JOURNAL*, 33: 219, 1928.
77. CHEN, K. K., AND MECK, W. J.: *THIS JOURNAL*, 28: 31, 1926.
78. FENG, C. T., AND READ, B. E.: *J. Am. Pharm. Assoc.*, 16: 1034, 1927.
79. PITTENGER, P. S.: *J. Am. Pharm. Assoc.*, 17: 634, 1928.
80. ROBBERS, H., AND GINADER, R.: *Klin. Wehnschr.*, 17: 528, 1938.
81. GADDUM, J. H.: *J. Physiol.*, 61: 141, 1926.
82. MONROE, R. R., AND DRELL, H. J.: *J. A. M. A.*, 135: 909, 1947.
83. ISBELL, H., WIKLER, A., EDDY, N. B., WILSON, J. L., AND MORAN, C. F.: *J. A. M. A.*, 135: 888, 1947.

A COMPARATIVE STUDY OF THE INTRAVENOUS PIGEON AND THE INTRAVENOUS CAT METHOD IN THE ASSAY OF DIGITALIS

HERBERT A. BRAUN AND LEHMAN M. LUSKY

*Division of Pharmacology, Food and Drug Administration,
Federal Security Agency, Washington 25, D. C.*

Received for Publication January 5, 1948

The assay of digitalis by the intravenous infusion in anesthetized cat as described in the U. S. Pharmacopoeia has been generally considered satisfactory. This method yields concordant and reproduceable results in the various laboratories where it is utilized (1). Gold and coworkers (2) have demonstrated that the results of the U.S.P. cat assay paralleled those of the human assay. However, it has become increasingly difficult to obtain cats and their cost is considerable.

In order to find a laboratory animal which might be substituted if necessary for the cat, the rabbit, guinea pig (3) and pigeon have been used in this laboratory. Of these, the pigeon has given the most satisfactory results. We were led to investigate the practicability of using this species for the assay of digitalis for several reasons. In the pigeon, we have an experimental animal which is abundant and readily available in all parts of this country and it is one of the least costly of all laboratory animals.

Hanzlik in 1929 (4) while working on pigeons demonstrated that the minimal fatal doses of the majority of his assays on various tinctures of digitalis agreed remarkably well when cats and pigeons were used.

In 1934, Haag and Woodley (5) described an intravenous pigeon method. They found that the minimal lethal dose per pigeon is 25% higher than that for the cat. They stated that the results of the pigeon method agreed favorably with those obtained by the Hatcher-Brody cat method.

Since the publication of the papers of Hanzlik and of Haag and Woodley the procedure for the standardization of digitalis has changed materially and the precision of the method has been increased.

Since the minimum lethal dose for the same preparation has been found to vary in different batches of cats the term "cat unit" is now seldom used as a criterion of digitalis activity. This variation led to the adoption of a standard, the U.S.P. Digitalis Reference Standard, (1942), which is now used for the determination of the relative potency of various digitalis products not only in the case of the cat method but also in methods using other species.

The accuracy of the method has been improved by calculating the injection rate on the basis of the weight of the animal. The rate of injection is unquestionably of great importance and contributes to the agreement in results which can now be obtained by various laboratories.

A more precise evaluation of the accuracy of this method has been obtained

by the application of statistical analysis to the results of the assay in order to determine the consistency of the animals utilized in the assay.

METHOD. The procedure which was used in the intravenous pigeon method was patterned after that described for the assay of digitalis in the U. S. Pharmacopoeia.

Adult pigeons of either sex weighing between 275 and 450 grams were selected at random. Birds which on gross examination appeared malnourished and emaciated were not used. In the course of this work three breeds of pigeons, namely, White Kings, Homers, and Common Barn Pigeons were utilized. No difference in susceptibility to digitalis was detected among these breeds. The pigeons were kept in the laboratory for a week before use on a commercial pigeon feed with an adequate supply of water and grit. The birds were fasted for 18 to 23 hours. On the day of the assay the pigeons were immobilized with ether, were accurately weighed and tied to suitable boards. The alar vein was exposed and cannulated. The cannula consisted of a blunted 22 gauge hypodermic needle which was inserted in the vein and tied into place. The arrangement was made to inject the appropriate test solution from a 10 cc. microburette calibrated to 0.1 cc. Very light ether anesthesia was maintained throughout the assay.

The solution to be assayed was diluted in such a way that the estimated fatal dose of the preparation per kilogram was diluted to 15 cc. with 0.9% sodium chloride solution. When the average death time fell outside of 60 to 90 minutes a new dilution was prepared. It should be noted here that since the pigeon is slightly more resistant to digitalis than the cat more tincture per 100 cc. will be required. We have found that for digitalis an additional one cc. of tincture per 100 cc. of test solution will suffice.

The diluted tincture was injected at a rate of 0.1 cc. per 100 grams of pigeon at 5 minute intervals until cardiac arrest supervened. Injections were made within a few seconds. Death in the pigeon as in the cat is of a typically circulatory nature. There is cardiac stoppage followed by dyspnoea, convulsions and death. The end point in pigeons is very sharp. At least six pigeons were used for each preparation to be assayed. We adhered to the U.S.P. requirement of a standard error of not over $\pm 5.7\%$. Six to eight pigeons were usually sufficient to come within this figure.

To date, we have completed the assays on 30 preparations of digitalis by both the intravenous pigeon method and the U.S.P. cat assay. Assays were made simultaneously by the two methods or at the most within two days of each other. When the sample was not already in liquid form enough tincture was prepared from it so that a single batch could be used for both the pigeon and the cat assays. For this study, we selected for assay at least one sample of the various types of *Digitalis purpurea* preparations on the American market. Included also are two preparations of *Digitalis lanata* which were assayed against the U.S.P. Digitalis Reference Standard (1942).

With the exception of Powdered leaves A and B, all the other preparations were samples collected as part of the regulatory activity under the Federal Food, Drug, and Cosmetic Act. Powdered leaf A is the International Standard (1936); Powdered leaf B is the New York Heart Association Powder No. 7. The latter is an atypical powder in that it yields a potency of 57% by the frog method, 62% by the intravenous guinea pig method and 85% by the U.S.P. cat method.

RESULTS. The results of the assays on the 30 preparations are summarized in Table I.

Inspection of the data in this series of assays reveals that the results obtained by one method are not consistently higher than the results obtained by the other method. Statistical analysis confirms this impression. This is in contrast to the data reported by Braun and Miller (6) in which the frog method of assay generally showed lower potencies than the cat method when the U.S.P. Reference Digitalis Powder was used as the standard.

TABLE I

A comparison of the potency estimates of various digitalis preparations by the pigeon and U.S.P. cat method

TYPE OF PREPARATION	PREPARATION	PERCENTAGE POTENCY	
		Pigeon Method	U.S.P. Cat Method
Digitalis purpurea			
Powdered Leaves	A	89	97
	B	85	85
	C	110	103
	D	117	105
	E	112	109
	F	102	92
	G	95	100
Capsules	A	104	118
	B	100	101
	C	108	108
	D (oil)	86	90
Tinctures	A	98	93
	B	77	85
	C	91	101
	D	134	120
Tablets	A	70	68
	B	104	96
	C	91	92
	D	89	97
	E	92	95
	F	95	107
	G	99	107
Enteric Coated Tablets	A	86	90
	B	65	63
Pills		90	94
Powdered Extract	A	70	73
	B	77	85
Purified glycosides		79	83
Digitalis lanata			
Powdered Leaves Tablets		236	250
		107	108

Table II gives the distribution of the percentage difference between the results obtained by the intravenous pigeon method and the U.S.P. cat method. Examination of this table reveals that in the majority of assays the differences

are $\pm 12\%$ or less. The exceptions are Capsule A in which the results differ by 13.6% and Tablet F in which the difference is 12.3% . Powder B which gave discordant results by the frog and the guinea pig methods showed perfect agreement between the pigeon and the U.S.P. cat methods of assay.

The data obtained in the present experiments suggest that the fatal dose of digitalis for pigeons may be less variable than that for cats. The fatal dose of the U.S.P. Digitalis Reference Standard (1942) determined on 60 pigeons was found to have a coefficient of variation of 10.4% while the comparable figure for 60 cats was 12.9% . This latter value is in surprisingly close agreement with the figure of 13.03% reported by de Lind van Wijngaarden (7).

In a series of 15 assays of the U.S.P. Digitalis Reference Standard (1942) completed on cats between December 1946 and July 1947, the minimal lethal dose varied from 74.25 to 99 mgm./kgm., a difference of 33% . For the pigeons, 10 assays of the U.S.P. Digitalis Reference Standard (1942) varied from 87.75 to 100.75 mgm./kgm., a difference of approximately 15% . In no case in which

TABLE II

Difference in percentage between the results of the pigeon and the U.S.P. cat assays

PERCENTAGE DIFFERENCE BETWEEN PIGEON AND U.S.P. CAT ASSAY RESULTS	NO. OF PREPARATIONS
0.0-2.0%	5
2.1-4.0%	4
4.1-6.0%	8
6.1-8.0%	1
8.1-10.0%	4
10.1-12.0%	6
12.1-14.0%	2
Over 14%	0

the U.S.P. Digitalis Reference Standard (1942) was assayed with pigeons was it necessary to use more than 6 birds to come within $\pm 5.7\%$ standard error requirement of the U.S.P. In the majority of assays of the U.S.P. Digitalis Reference Standard (1942) on cats more than 6 cats were needed to yield this figure. It would seem from these data that not only are the results of the separate assays on pigeons more consistent but various batches of pigeons vary less than various batches of cats.

The average number of milligrams required for the U.S.P. Digitalis Reference Standard (1942) on pigeons was 96.22 mgm./kgm. as contrasted with 84.83 mgm./kgm. for the cat assay, a difference of 13.4% . Haag and Woodley (5) have reported a 25% difference using the Hatcher-Brody method.

DISCUSSION. Statistical analysis of the potencies of digitalis preparations as determined by the cat and pigeon methods reveals that there are no significant differences between them. Since Gold and coworkers (2) have demonstrated that the results of the U.S.P. cat method paralleled assays on humans, it is to be expected that the results of the pigeon method would show a similar agreement with the human assay.

One of the advantages enjoyed by the cat method over other biological methods is the simplicity of the technique involved. It can be stated that the technique of the intravenous pigeon method is no more difficult or time-consuming than the present U.S.P. method.

Perhaps the most common objection voiced to the performance of the cat method is the present scarcity of cats in certain sections of this country. In the pigeon we have an experimental animal which is abundantly distributed and available in all parts of the United States.

The pigeon is one of the cheapest of all experimental animals; in fact, the cost of the intravenous pigeon assay is only a fraction of the cost of the cat assay. At the present price of pigeons we are able to do a complete assay on a preparation for less than the cost of a single cat.

CONCLUSIONS

1. A method for the assay of digitalis by intravenous infusion into pigeons is described. It is based on the present U.S.P. method for the assay of Tincture of Digitalis.

2. The potency estimates of 30 digitalis products obtained by this method varied in no case by more than 13.6% from those obtained by the present U.S.P. method. The average deviation between the two methods was not significantly different from zero.

3. The coefficient of variation of the lethal dose of digitalis for the pigeon in our tests was 10.4% as contrasted to 12.9% for the cats.

4. Preliminary investigation reveals that not only are pigeons more consistent in a given assay than are cats but various batches of pigeons seem to vary less from each other than various batches of cats.

We are greatly indebted to Dr. B. J. Vos for his interest and helpful criticism.

REFERENCES

- (1) BLISS, C. I.: J. Amer. Pharmaceu. Assoc., 33: 225, 1944.
- (2) GOLD, H., CATTELL, McK., KWIT, N. AND KRAMER, M.: This Journal, 73: 212, 1941.
- (3) BRAUN, H. A. AND SIEGFRIED, A.: J. Amer. Pharmaceu. Assoc., 36: 363, 1947.
- (4) HANZLIK, P.: This Journal, 35: 363, 1929.
- (5) HAAG, H. B., AND WOODLEY, J. D.: This Journal, 51: 360, 1934.
- (6) BRAUN, H. A. AND MILLER, L. C.: J. Amer. Pharmaceu. Assoc., 31: 225, 1942.
- (7) DE LIND VAN WIJNGAARDEN, C.: Arch. f. exp. Path. u. Pharmacol., 113: 40, 1926.

RELATIVE EXPERIMENTAL CORONARY VASODILATOR POTENCIES AND TOXICITIES OF PAPAVERINE, THEOPHYLLINE, AND A PAPAVERINE-THEOPHYLLINE MOLECULAR ASSOCIATION COMPOUND

CLAUDE V. WINDER AND MARGARET E. KAISER

Research Laboratories, Parke, Davis and Company, Detroit, Michigan

Received for Publication January 7, 1948

In view of current interest in papaverine and theophylline preparations in therapy of coronary disease, a molecular association compound (1, 2) of the two basic drugs, hereafter called D-104, was subjected to pharmacological investigation. Questions needing study concerned possibilities of mutual facilitation by the two moieties in coronary vasodilatation, of an advantageous combined pattern of side actions, and of a more elastic therapeutic range. Actually, it soon became apparent that the compound's main interest lay in its provision of a somewhat unique circle of comparisons of its constituents, rather than in its own therapeutic promise.

METHODS. The quickly isolated, beating rabbit heart was perfused via the aorta against closed aortic valves (method of Langendorff), with Ringer-Locke solution (0.03% NaHCO_3) saturated with oxygen and maintained at constant temperature ($36^\circ\text{--}38^\circ\text{C.}$) and pressure (ca. 71 cm.). Ventricular contractions were recorded kymographically. The rate of coronary perfusion was measured by collection of the outflow in a graduated cylinder for one-minute periods, recorded at intervals of 1.5 minutes on the kymographic time record. In one of the experiments the left ventricle was incised to allow escape of small fluid accumulations and in two experiments such accumulations were drained away separately through a small steel cannula and light tubing (3). A negligible Thebesian return and variable aortic valvular leakage are represented in such left-side accumulations. Injections of the warmed drug solutions were made with a small syringe and needle through the wall of a short segment of rubber tubing connecting the cannula with the system. Solutions were rendered equivalent in total ionic concentration by means of NaCl.

The ratio of the dilating potency of one drug to that of another was computed as the reciprocal ratio of respective doses required to give equal peak increases in flow (response ratios of unity). Two drugs were injected alternately. The response ratios were calculated by comparing an actual response to one drug, A, with a hypothetical response to the other, B, arrived at by interpolation over time between an actual response to B just preceding and one just following A, with alternation in status of the drugs in the calculations. In this manner degradation in sensitivity of the preparation and non-linearity of such degradation were controlled. While valvular leakage might have altered the dose ratio-response ratio slope, hence precision of comparison in the experiments where it was not collected separately, it would not be expected to have *biased* the statistical comparison at unity response ratio. Results are derived from 70 injections in 6 preparations. Peak increases in flow ranged from 0.5 to 11.2 cc./min., mean 5.2 (4.1 to 165 per cent, mean 48.7 per cent of current basal flow rates).

Groups of white mice (ca. 20 gm. body weight) were injected intraperitoneally with graded doses of 2 per cent solutions of D-104 or of papaverine hydrochloride for comparison of acute lethal toxicities.

Dogs anesthetized with phenobarbital were used for comparison of the acute cardiovas-

cular and respiratory effects of the drugs. Femoral arterial pressure was recorded with the conventional mercury manometer. Respiratory rate and a qualitative index of changes in depth (tracheal air velocity) were recorded by means of a side-tube arrangement (modification of Pfeiffer and Moore's (4) device). Heart rate was recorded electrically with a Junior Garceau Electroencephalograph fitted with a special voltage adaptor.¹

RESULTS. I. *Relative coronary vasodilatation potencies.*

A. *D-104 vs. theophylline.* From semi-quantitative preliminary experiments with aminophylline (U.S.P.) vs. D-104 it was estimated that 10 to 20 times as much theophylline as D-104 would be required for equivalent increases in perfusion flow through the coronary bed of the isolated heart. Therefore 20 $\mu\text{gm.}$ of D-104 and 400 $\mu\text{gm.}$ theophylline (U.S.P.) were alternately injected. Recorded effects on the myocardium were very small or absent.²

The (logarithmic) mean perfusion response-ratio was 0.93 at this dose-ratio of 1 to 20 (D-104 relative to theophylline). The 95% confidence range of the ratio was 0.73 to 1.18. Doubling the amount of D-104 (dose-ratio of 1 to 10) yielded a response-ratio of 1.21 suggesting sensitivity of the preparation to dosage. That D-104 is many times as potent as its one constituent, theophylline, was thus confirmed. As a rough estimate from such an experiment, the potency-ratio would lie between 10 and 20 by weight, or *ca.* 25 and 50 on a molecular basis.

The question arises as to whether these results arose from mutual potentiation of theophylline and papaverine in the compound, or from papaverine being many times as potent as the theophylline. The first test of these alternatives was a comparison of D-104 with its other constituent, papaverine.

B. *D-104 vs. papaverine hydrochloride.* In 3 experiments, 21 response-ratios corresponding to dose-ratios of 0.7 to 1.7 as much D-104 as papaverine hydrochloride (6-138 $\mu\text{gm.}$ D-104, 4-200 $\mu\text{gm.}$ papaverine HCl) were available. Of these, the group (logarithmic) means of 17, corresponding to dose-ratios from 1.1 to 1.7, arranged themselves linearly on logarithmic co-ordinate paper, crossing the response-ratio ordinate of unity.³ Analysis of this regression of logarithms of response-ratios on logarithms of dose-ratios yielded (antilogarithmically) a dose-ratio of 1.5 as much D-104 as papaverine hydrochloride required for equal responses, with a 95% confidence range (5, 6) of 1.4 to 1.8. Since the molecular weight of D-104 relative to that of papaverine hydrochloride is 1.4, this constitutes a strong suggestion (*ca.* 5% level of significance) that the action of D-104 might be somewhat less than expected on the basis of its papaverine content, and very probably not more. The likelihood of mutual potentiation between

¹ The Electro-Medical Laboratory, Holliston, Mass.

² Under the conditions employed in these experiments the hearts were distinctly hypodynamic and excessive doses of the drugs were avoided. Any difference in slight direct myocardial actions of the drugs, expected under such refractory myocardial conditions, was rendered indefinite and perhaps masked by indirect effects of improved flow. Such circumstances are not without advantage in an *in vitro* analysis of vascular effects.

³ This analysis implies parallel, linear log dose-log. peak-response relationships for the two drugs being compared. In an analogous but much more intensive comparison (46 hearts) of papaverine and another drug, to be reported, this hypothesis has been found to hold within limits of error.

RELATIVE EXPERIMENTAL CORONARY VASODILATOR POTENCIES AND TOXICITIES OF PAPAVERINE, THEOPHYLLINE, AND A PAPAVERINE-THEOPHYLLINE MOLECULAR ASSOCIATION COMPOUND

CLAUDE V. WINDER AND MARGARET E. KAISER

Research Laboratories, Parke, Davis and Company, Detroit, Michigan

Received for Publication January 7, 1948

In view of current interest in papaverine and theophylline preparations in therapy of coronary disease, a molecular association compound (1, 2) of the two basic drugs, hereafter called D-104, was subjected to pharmacological investigation. Questions needing study concerned possibilities of mutual facilitation by the two moieties in coronary vasodilatation, of an advantageous combined pattern of side actions, and of a more elastic therapeutic range. Actually, it soon became apparent that the compound's main interest lay in its provision of a somewhat unique circle of comparisons of its constituents, rather than in its own therapeutic promise.

METHODS The quickly isolated, beating rabbit heart was perfused via the aorta against closed aortic valves (method of Langendorff), with Ringer-Locke solution (0.03% NaHCO_3) saturated with oxygen and maintained at constant temperature ($36^\circ\text{--}38^\circ\text{C}$) and pressure (ca 71 cm). Ventricular contractions were recorded kymographically. The rate of coronary perfusion was measured by collection of the outflow in a graduated cylinder for one-minute periods, recorded at intervals of 15 minutes on the kymographic time record. In one of the experiments the left ventricle was incised to allow escape of small fluid accumulations and in two experiments such accumulations were drained away separately through a small steel cannula and light tubing (3). A negligible Thebesian return and variable aortic valvular leakage are represented in such left side accumulations. Injections of the warmed drug solutions were made with a small syringe and needle through the wall of a short segment of rubber tubing connecting the cannula with the system. Solutions were rendered equivalent in total ionic concentration by means of NaCl.

The ratio of the dilating potency of one drug to that of another was computed as the reciprocal ratio of respective doses required to give equal peak increases in flow (response ratios of unity). Two drugs were injected alternately. The response ratios were calculated by comparing an actual response to one drug, A, with a hypothetical response to the other, B, arrived at by interpolation over time between an actual response to B just preceding and one just following A, with alternation in status of the drugs in the calculations. In this manner degradation in sensitivity of the preparation and non linearity of such degradation were controlled. While valvular leakage might have altered the dose response ratio slope, hence precision of comparison in the experiments where it was not collected separately, it would not be expected to have biased the statistical comparison at unity response ratio. Results are derived from 70 injections in 6 preparations. Peak increases in flow ranged from 0.5 to 11.2 cc/min, mean 5.2 (4.1 to 16.5 per cent, mean 48.7 per cent of current basal flow rates).

Groups of white mice (ca 20 gm body weight) were injected intraperitoneally with graded doses of 2 per cent solutions of D-104 or of papaverine hydrochloride for comparison of acute lethal toxicities.

Dogs anesthetized with phenobarbital were used for comparison of the acute cardiovas-

cular and respiratory effects of the drugs. Femoral arterial pressure was recorded with the conventional mercury manometer. Respiratory rate and a qualitative index of changes in depth (tracheal air velocity) were recorded by means of a side-tube arrangement (modification of Pfeiffer and Moore's (4) device). Heart rate was recorded electrically with a Junior Garceau Electroencephalograph fitted with a special voltage adaptor.¹

RESULTS. 1. *Relative coronary vasodilatation potencies.*

A. *D-104 vs. theophylline.* From semi-quantitative preliminary experiments with aminophylline (U.S.P.) vs. D-104 it was estimated that 10 to 20 times as much theophylline as D-104 would be required for equivalent increases in perfusion flow through the coronary bed of the isolated heart. Therefore 20 μ gm. of D-104 and 400 μ gm. theophylline (U.S.P.) were alternately injected. Recorded effects on the myocardium were very small or absent.²

The (logarithmic) mean perfusion response-ratio was 0.93 at this dose-ratio of 1 to 20 (D-104 relative to theophylline). The 95% confidence range of the ratio was 0.73 to 1.18. Doubling the amount of D-104 (dose-ratio of 1 to 10) yielded a response-ratio of 1.21 suggesting sensitivity of the preparation to dosage. That D-104 is many times as potent as its one constituent, theophylline, was thus confirmed. As a rough estimate from such an experiment, the potency-ratio would lie between 10 and 20 by weight, or *ca.* 25 and 50 on a molecular basis.

The question arises as to whether these results arose from mutual potentiation of theophylline and papaverine in the compound, or from papaverine being many times as potent as the theophylline. The first test of these alternatives was a comparison of D-104 with its other constituent, papaverine.

B. *D-104 vs. papaverine hydrochloride.* In 3 experiments, 21 response-ratios corresponding to dose-ratios of 0.7 to 1.7 as much D-104 as papaverine hydrochloride (6-138 μ gm. D-104, 4-200 μ gm. papaverine HCl) were available. Of these, the group (logarithmic) means of 17, corresponding to dose-ratios from 1.1 to 1.7, arranged themselves linearly on logarithmic co-ordinate paper, crossing the response-ratio ordinate of unity.³ Analysis of this regression of logarithms of response-ratios on logarithms of dose-ratios yielded (antilogarithmically) a dose-ratio of 1.5 as much D-104 as papaverine hydrochloride required for equal responses, with a 95% confidence range (5, 6) of 1.4 to 1.8. Since the molecular weight of D-104 relative to that of papaverine hydrochloride is 1.4, this constitutes a strong suggestion (*ca.* 5% level of significance) that the action of D-104 might be somewhat less than expected on the basis of its papaverine content, and very probably not more. The likelihood of mutual potentiation between

¹ The Electro-Medical Laboratory, Holliston, Mass.

² Under the conditions employed in these experiments the hearts were distinctly hypodynamic and excessive doses of the drugs were avoided. Any difference in slight direct myocardial actions of the drugs, expected under such refractory myocardial conditions, was rendered indefinite and perhaps masked by indirect effects of improved flow. Such circumstances are not without advantage in an *in vitro* analysis of vascular effects.

³ This analysis implies parallel, linear log. dose-log. peak-response relationships for the two drugs being compared. In an analogous but much more intensive comparison (46 hearts) of papaverine and another drug, to be reported, this hypothesis has been found to hold within limits of error.

the two constituents of D-104 thus disappeared, leaving the alternative that papaverine is many times more potent than theophylline; in fact, the contribution of theophylline to the action of the complex, at the doses used, was inappreciable or even subtractive. Effects on recorded ventricular contractions were similar for the 2 drugs, and usually slight or absent.

C. Theophylline (U.S.P.) vs. papaverine hydrochloride. The next step was to check, directly, the third side of the triangle of comparisons, theophylline relative to papaverine. This experiment was designed to provide a total of 8 response-ratios, corresponding to dose-ratios of 14.3, 28.6, and 57.1 as much theophylline as papaverine hydrochloride (400 μ gm. theophylline, 7-28 μ gm. papaverine hydrochloride). Myocardial effects were slight. While the 3 group means did not fall so strikingly on a linear (logarithmic) course as in the preceding case, the deviation was not statistically significant ($F = 5.26$, 1 and 5 degrees of freedom (6)), and analysis of linear (logarithmic) regression provided essentially the same slope as in the preceding comparison. The analysis yielded a dose-ratio, by weight, of 17.9 (best estimate) as much theophylline as papaverine hydrochloride required for equivalent peak dilatation. The 95% confidence range was 7.7 to 25.2. On a molecular basis, this range corresponds to 15 to 48, not in disagreement with the molecular potency-ratio range more roughly estimated from the comparison of D-104 with theophylline. The conclusion that the activity of papaverine in D-104 was not demonstrably supplemented by the theophylline moiety present, and more specifically, that papaverine hydrochloride was many times as potent as theophylline, was thus confirmed.

D. D-104 vs. an equivalent mixture of papaverine hydrochloride and theophylline. To test the possible influence of the loose molecular association in D-104 as supplied and/or to control the rationale of the technique, in the last experiment D-104 was compared with an equimolecular mixture of theophylline and papaverine hydrochloride. Eleven response ratios were available, corresponding to equivalent-dose ratios of 0.77, 0.97, and 1.16. Actual doses were 50 μ gm. D-104 and, in equivalents of D-104, 0.77 to 1.16 as much of the mixture. Effects on ventricular contractions were slight, and indistinguishable for the two solutions. Analysis of the (statistically significant) regression of log.-response ratios on log.-dose ratios, as above, yielded a dose ratio of 1.00, or precisely molecularly equivalent amounts of the two solutions for equivalent responses. The 95% confidence range was 0.94 to 1.08. Thus, no detectable difference was demonstrated between the molecular association of papaverine with theophylline on the one hand, and an equivalent mixture of its constituents on the other. At the same time, interesting precision of the technic when used in comparing *qualitatively* similar materials was indicated.

The pH of the solutions of D-104 was from 3.7 to 4.1; that of theophylline, 5.6; papaverine hydrochloride, 5.1 to 5.2; the theophylline-papaverine hydrochloride mixture, 4.8. If the greater acidity of D-104 should have contributed to its high coronary-dilator potency relative to theophylline (section IA, above) it should probably also have contributed to its potency relative to papaverine (IB, above); yet in the latter case it was somewhat less, rather than more potent,

on a molecular basis, than papaverine. Thus, the much smaller pH differences between the papaverine and theophylline, at a level nearer neutrality, would hardly be expected to affect the papaverine-theophylline potency ratio as determined (IC, above). Furthermore, precise equality of potencies of D-104 and the equivalent mixture of papaverine and theophylline was demonstrated in spite of considerable difference in pH of the respective solutions. Thus, in the small volumes injected into the buffered perfusion fluid, acidity of the drug solutions did not demonstrably modify the results.

II. *Relative cardiovascular and respiratory effects.* At molecular equivalents, and dose levels represented by 0.2 to 9.3 mgm./kgm. of papaverine hydrochloride quickly injected, no significant difference between D-104 and papaverine hydrochloride could be detected in the cardiovascular and respiratory effects. Typical papaverine responses were apparent in each case, as illustrated in figure 1. Heart rate with either preparation was accelerated as much as 5 to 20%, somewhat after the peak of the blood-pressure and respiratory effects. Such heart rate changes were probably at least largely secondary to the pressure and respiratory effects.

Thus, as in the isolated heart, at effective dose levels the actions of D-104 were accountable in terms of its papaverine content. No theophylline contribution was evident.

Ten or 18 times as much aminophylline by weight, containing 16 or 30 times as many equivalents of theophylline, respectively, caused much more severe vascular depression and a different quality of respiratory stimulation than a given dose of papaverine, as also illustrated in Figure 1. Cardiac acceleration was greater. Since this dose ratio is conservative relative to that found for theophylline vs. papaverine in coronary dilatation (15-48), it would appear that papaverine may be at a therapeutic advantage.

III. *Relative acute lethal toxicities in white mice.* The intraperitoneal median lethal doses in white mice, computed from the data by the method of maximum likelihood (7, 5), were 129 mgm./kgm. (95% limits, 113 and 149) for papaverine hydrochloride, and 150 mgm./kgm. (95% limits, 129 and 177) for D-104. The latter, expressed as papaverine hydrochloride for comparison on a molecular basis, converts to 109 (95% limits, 93 and 128). The slopes of regression of probits kill on log. dose were not significantly different (7.28 for papaverine hydrochloride and 7.43 for D-104). This means that the inter-animal variabilities in susceptibility to the respective drugs were not significantly different.

The relative molecular toxicity of D-104, referred to papaverine hydrochloride, with pooled slopes and variance used in the computations (5), was 1.19, with 95% limits of 0.98 and 1.45. That is, there is a suggestion that at lethal dose levels of D-104, the theophylline moiety added to the terminal pharmacodynamic effects of papaverine.

Discussion. No therapeutic advantage of a molecular association of theophylline and papaverine was suggested.

Experimental evidence that papaverine is capable of significant dilatation of coronary vessels is now considerable and varied. Macht (8) first demonstrated



200 MGM. AMINOPHYLLIN



28 MGM. D-104



20 MGM. PAPAVERINE-HCl



360 MGM. AMINOPHYLLIN

FIG. 1. DOG, ANESTHETIZED WITH SODIUM PHENOBARBITAL INTRAPERITONEALLY. Femoral arterial blood pressure, respiration, and time in minutes. Rapid femoral intravenous injections with syringe and needle

its relaxant action on excised coronary-artery rings (pig and human). He and others have observed its ability to increase the rate of perfusion, at constant pressure, of the vascular bed of isolated hearts of animals (8, 9, 10, 11, 12, 13), and man (11). Anrep (14) observed it to cause "considerable" increase in the

coronary circulation of the human heart-lung preparation. Various controls have indicated the increased flow to be independent of changes in myocardial mechanics, and presumably a direct one on coronary vessels: viz., its occurrence in quiescent animal (9) and human (11) hearts, in hearts in strophanthin arrest (11, 12), and in constantly fibrillating hearts (13). That its action is sufficiently selective to cause increased coronary flow in the intact organism in spite of more or less general vasodilatation was shown presumptively by Macht (8), but more certainly by Essex et al. in the unanesthetized animal (15) and Eckenhoff and Hafkenschiel (16) in the anesthetized animal. Its administration is reported to diminish electrocardiographic evidence of myocardial anoxia following coronary ligation, with or without superimposed anoxemia (17). Mokotoff and Katz (18) have recently demonstrated the ability of the drug to reduce the size of experimental myocardial infarction.

Comparisons of papaverine with the better known and still more commonly used xanthines are, however, few. Trussewitsch (11) found papaverine at 1:100,000 concentration a more reliable dilator than caffeine at 1:1000 in the human hearts. One observation with 1:10,000 theobromine was positive. Lindner and Katz (13) apparently obtained greater and more durable increases in flow in fibrillating dog hearts with 16-33 mgm. of papaverine hydrochloride than with 24-48 mgm. aminophylline or 125-500 mgm. caffeine sodium benzoate. Essex et al. (15) obtained roughly similar increases in coronary flow in the intact dog with 1.25 and 1.64 mgm./kgm. of papaverine (2 observations) and 12 to 32 mgm./kgm. of aminophylline. Eckenhoff and Hafkenschiel (16) employed papaverine doses of 0.004 to 0.040 mgm. intra-arterially (coronary) and 15 to 20 mgm. intravenously, and aminophylline doses of 1.0 mgm. intra-arterially and 60 to 120 mgm. intravenously. As regards comparative effects, the aminophylline doses were apparently excessive relative to papaverine's. Whereas 3 to 10 mgm./kgm. of papaverine hydrochloride reduced ECG evidence of myocardial anoxia in the experiments of Leslie and Mulinos (17), 5 to 15 mgm./kgm. of aminophylline was without demonstrated effect. Mokotoff and Katz (18) found significantly more reduction of experimental infarct size with 5 mgm./kgm. doses of papaverine hydrochloride than with 15 mgm./kgm. doses of aminophylline. Thus, all six sets of experiments point up papaverine's superior potency, but a quantitative statement cannot readily be made from them. The present experiments may be regarded as a contribution particularly to the quantitative aspects of this problem.

In view of its relative efficiency (experimental coronary-dilator potency relative to toxicity), together with relative absence of myocardial-stimulant liability and presence of certain properties directable against arrhythmias and fibrillation (10, 19, 20, 21, 22), it is not surprising that papaverine should be responsible for some increased hope of effective medication in coronary disease, particularly since the vigorous work of Katz and associates (13, 18, 19, 20, 23).

SUMMARY

In the isolated rabbit heart, a molecular association compound of papaverine and theophylline was precisely equal in coronary-dilating potency to an equiva-

lent equimolecular mixture of papaverine hydrochloride and theophylline. Papaverine hydrochloride was 18, ± 7 or -10 (95% confidence limits), times as potent as theophylline on a weight basis. On a molecular basis, theophylline was relatively so impotent that its contribution in the molecular association compound could not be detected, either on the coronary vascular bed, or in cardiovascular and respiratory effects in the anesthetized dog, by molecular comparison of the compound with papaverine hydrochloride. There was, indeed, a strong suggestion ($P = ca. 0.05$) that the presence of theophylline (competitively?) diminished papaverine's actions in the isolated heart.

However, at lethal dose levels (white mice), there was some suggestion ($P = > 0.05$) that theophylline was beginning to contribute slightly to toxicity.

Acknowledgement. The authors are indebted to E. Lyons, formerly of these laboratories, who prepared D-104, and to C. C. Pfeiffer, now of the University of Illinois, whose initiating preliminary experiments, subsequently confirmed, indicated it to be several times as potent as aminophylline.

REFERENCES

1. MOSSINI, A.: *Boll. chim. farm.*, **78**: 261, 1939. (Ref. from *Chem. Abstr.*, **33**: 7733, 1939).
2. SINGER, R.: *Wien. klin. Wchnschr.*, **47**: 1353, 1934.
3. WIGGERS, C. J.: *Am. J. Physiol.*, **24**: 391, 1909.
4. PFEIFFER, C. C., AND MOORE, V. A.: *J. Lab. & Clin. Med.*, **30**: 184, 1945.
5. BLISS, C. I.: *Biometrics Bull.*, **1**: 57, 1945.
6. SNEDECOR, G. W.: *Statistical Methods*, 4th Ed., The Collegiate Press, Inc., Ames, Iowa, 1946.
7. BLISS, C. I.: *Quart. J. Pharm. & Pharmacol.*, **11**: 192, 1938.
8. MACHT, D. I.: *J. A. M. A.*, **64**: 1489, 1915.
9. GRUBER, C. M., AND ROBINSON, P. I.: *This Journal*, **37**: 429, 1929.
10. SAKUSSOW, W. W. jun.: *Arch. f. exper. Path. u. Pharmacol.*, **144**: 331, 1929.
11. TRUSSEWITSCH, B. I.: *Ztschr. f. d. ges. exper. Med.*, **75**: 167, 1931.
12. MULINOS, M. G., SHULMAN, I., AND POMERANTZ, L.: *This Journal*, **66**: 27, 1939.
13. LINDNER, E., AND KATZ, L. N.: *This Journal*, **72**: 306, 1941.
14. ANREP, G. V.: *Stanford University Publications, University Series, Medical Sciences*, **3**: 73, Stanford University Press, Stanford University, 1936.
15. ESSEX, H. E., WEGRIA, R. G. E., HERRICK, J. F., AND MANN, F. C.: *Am. Heart J.*, **19**: 554, 1940.
16. ECKENHOFF, J. E., AND HAFKENSCHIEL, J. H.: *This Journal*, **91**: 362, 1947.
17. LESLIE, A., AND MULINOS, M. G.: *Am. Heart J.*, **24**: 679, 1942.
18. MOKOTOFF, R., AND KATZ, L. N.: *Am. Heart J.*, **30**: 215, 1945.
19. LINDNER, E., AND KATZ, L. N.: *Am. J. Physiol.*, **133**: 155, 1941.
20. ELEK, S. R., AND KATZ, L. N.: *This Journal*, **74**: 335, 1942.
21. WEGRIA, R., AND NICKERSON, N. D.: *This Journal*, **75**: 50, 1942.
22. McEACHERN, C. G., SMITH, F. H., AND MANNING, G. W.: *Am. Heart J.*, **21**: 25, 1941.
23. ELEK, S. R., AND KATZ, L. N.: *J. A. M. A.*, **120**: 434, 1942.

THE EFFECT OF DITHIOBIURET (DTB) ON THE ELECTROLYTE AND WATER CONTENT OF SKELETAL MUSCLE, AND ON CARBOHYDRATE METABOLISM¹

SAM SEIFTER, DAVID M. HARKNESS, EDWARD MUNTWYLER, AND JOSEPH SEIFTER

Department of Biochemistry, Long Island College of Medicine, Brooklyn, and the Wyeth Institute of Applied Biochemistry, Philadelphia

Received for publication January 9, 1948

Astwood (1) and Astwood, Hughes and co-workers (2) have reported that the administration of dithiobiuret (DTB)— $(\text{NH}_2 \cdot \text{C}(=\text{S}) \cdot \text{NH} \cdot \text{C}(=\text{S}) \cdot \text{NH}_2)$ to rats in their drinking water as a 0.002% solution produced a flaccid paralysis involving the muscles of the entire body with the exception of the muscles of respiration and those of the head and neck. If the concentration of DTB was reduced to 0.001% the animals were unaffected. These workers concluded that the administration of DTB caused a reversible paralysis in rats which did not seem to be due to a disturbance of the muscles themselves, of the peripheral nerves, or of the myoneural junction.

DuBois and his co-workers (3, 4) have studied the effects on carbohydrate metabolism of alpha-naphthylthiourea (ANTU), allylthiourea, phenylthiourea, and thiourea, the chemical structures of which are closely related to that of DTB. They found that ANTU and phenylthiourea possessed a marked hyperglycemic action when administered to rats in amounts as low as 10 mg. per kilogram. The hyperglycemic actions of allylthiourea and thiourea were much less pronounced and large doses of these compounds were necessary to elicit a response. Animals receiving any of these hyperglycemic agents showed a depletion of liver glycogen. The latter authors also found that the hyperglycemia resulting from administration of ANTU was antagonized by insulin and ergotamine and completely prevented by adrenal-demedullation. Animals treated with ANTU, however, exhibited no abnormality in the oxidation of glucose, and an increased amount of epinephrine could not be detected in the blood.

The investigation reported here was undertaken (a) to determine if the paralysis produced by DTB is accompanied by changes in the chemical composition of skeletal muscle, and (b) to determine if DTB causes a disturbance in carbohydrate metabolism.

METHODS AND RESULTS. *Effect of DTB on the Electrolyte Composition of the Skeletal Muscle of Rats.* Male rats of the white Wistar strain, weighing between 200 and 300 grams each, were placed on a constant diet consisting of Purina Dog Chow for a period of one week. At the end of this period certain of the animals were given DTB² in the drinking water as a

¹ A preliminary report of this work was presented before the American Society of Biological Chemists, Chicago, Illinois, May 18, 1947 (Federation Proceedings 6, 289, 1947), and was aided by a grant from Wyeth Incorporated, Philadelphia.

² The dithiobiuret was kindly supplied by the American Cyanamid Company.

0.002% solution, while others served as controls. Food was allowed *ad libitum*. With this concentration of DTB the animals exhibited, in from 5 to 8 days, a paralysis which generally but not always affected the hind quarters. When the paralysis had become severe the animals were placed under sodium pentobarbital anesthesia and arterial blood was collected under oil by direct puncture of the left ventricle. The gastrocnemius muscles were then removed and submitted to analysis for water, chloride, sodium, potassium, total and acid soluble phosphorus, creatine, and total neutral fat. The methods used for these determinations were the same as reported elsewhere (5) excepting that the method of Lowry and Hastings (6) was employed for the extraction of the electrolytes and for the determination of chloride.

The results of these analyses are shown in Table I. For purposes of comparison the data obtained on skeletal muscle are expressed in terms of 100 grams of fat-free solids. It may be seen that there was no striking difference between the control and experimental groups. The slightly higher creatine level found in

TABLE I

Chemical determinations on skeletal muscle of normal rats and of rats paralyzed by dithiobiuret (Muscle values expressed per 100 gm. fat-free solids)

	NORMAL RATS (5)*		PARALYZED RATS (8)*	
	Mean	Range	Mean	Range
Water, gm.	327.0	324-331	330.1	319-359
Chloride, m.eq.	5.0	4.7-5.2	5.2	4.7-6.5
Potassium, m.eq.	48.9	47.5-50.2	48.1	46.3-50.6
Sodium, m.eq.	7.4	7.2-7.6	8.3	7.4-8.7
Total phosphorus, mM.	32.5	31.2-33.5	31.6	30.8-32.1
Acid soluble phosphorus, mM.	27.0	26.5-27.4	26.5	25.2-27.6
Creatine, mg.	2256	2130-2350	2462	2310-2720
Plasma chloride, m.eq. per 1000 gm. water	103.2	101-105	106.9	104-113

* The figures within the parentheses indicate the number of animals.

the experimental group can probably be attributed to inanition, since the animals receiving the DTB showed an immediate decrease in food consumption and lost weight rapidly.

Effect of DTB on the Electrolyte Composition of the Skeletal Muscle of Rabbits. Male and female rabbits weighing between 2 and 4 kilograms were employed. The animals were maintained on a diet of Purina Rabbit Chow, and those which served as controls were paired during the experimental period. The DTB was administered daily by subcutaneous injection of an aqueous solution in an amount of 7 mg. per kilogram of body weight. It is of interest to note that immediately after receiving the first injection the animals ceased eating and generally showed evident difficulty of voiding urine during the remainder of the experimental period. Upon autopsy of the animals it was found that the bladder was markedly distended, and in one case this organ contained as much as 300 ml. of urine. Usually the stomachs were filled with food although the animals generally had eaten comparatively little since the first experimental day.

A marked paralysis involving the skeletal muscles was encountered in from 6 to 12 days. When paralysis was severe, arterial blood and skeletal muscle

(lumbar portion of the sacrospinalis) were obtained under sodium pentobarbital anesthesia. Heparinized plasma, separated under oil, was analyzed for water, chloride, carbon dioxide content, sodium, potassium, and, in a few instances for calcium. Although no tabulated data are presented, suffice it to state that by comparison with the control animals no significant changes of the carbon dioxide content, sodium, potassium or calcium levels of the plasma of the paralyzed rabbits were encountered. The muscle samples were subjected to the same analyses as was the rat muscle. The results of the analysis of the muscles of three rabbits with severe paralysis and of three pair-fed control animals are presented in Table II. As in the case of the rats studied, the data obtained with the rabbits receiving DTB showed no striking differences when compared with those for the pair-fed controls.

TABLE II

Chemical determinations on skeletal muscle of pair-fed control rabbits and of rabbits paralyzed by dithiobiuret
(Muscle values expressed per 100 gm. fat-free solids)

	PAIR-FED CONTROLS (3)*	PARALYZED RABBITS (3)*
	mean values	
Water, gm.....	353.7	325.0
Chloride, m.eq.....	4.4	3.9
Potassium, m.eq.....	48.9	44.1
Sodium, m.eq.....	8.7	8.0
Total phosphorus, mM.....	34.6	33.9
Acid soluble phosphorus, mM.....	31.2	30.6
Creatine, mg.....	3070	3010
Plasma chloride, m.eq. per 1000 gm. water ..	107.4	113.0

* The figures within the parentheses indicate the number of animals.

It is apparent, therefore, that the paralysis produced by DTB in rabbits and rats is unaccompanied by any significant changes in the skeletal muscle content of water, chloride, sodium, potassium, phosphorus, and creatine.

Effect of DTB on the Blood Glucose of Rabbits. In experiments dealing with the effect of DTB on the blood glucose concentration of rabbits, the substance was administered by subcutaneous injection at three levels: 7, 40 and 100 mg. per kilogram of body weight. The two lower dosages were given as an aqueous solution, while at the 100 mg. level the DTB was administered in propylene glycol. Preliminary experiments had indicated that propylene glycol *per se* in the amounts employed did not produce any of the changes reported here. Blood glucose determinations were made hourly on samples obtained from the marginal ear vein, using the Jeghers-Myers modification of the Folin-Malmros method (7).

It was noted that a single injection of 100 mg. of DTB per kilogram of body weight resulted in an immediate and significant rise in the blood sugar, whereas a single injection of DTB at either of the two lower dosage levels was virtually without effect on the blood sugar concentration. However, the prolonged administration of the compound at a level of 7 mg. per kilogram was sometimes

found to be accompanied by a moderate increase in the blood glucose level. In one case a value of 220 mg. of glucose per 100 ml. of blood was obtained with an animal which had received this dosage of DTB for a period of 12 days and which was moribund.

Because the animals receiving the repeated doses of the compound stopped eating almost immediately after the first injection, and thus at the time of the glycemic changes were apparently in a state of depletion with respect to glycogen, experiments were undertaken with rabbits which had been starved for 3 days. These animals could be considered as having a nutritional status similar to that of the chronically intoxicated rabbits.

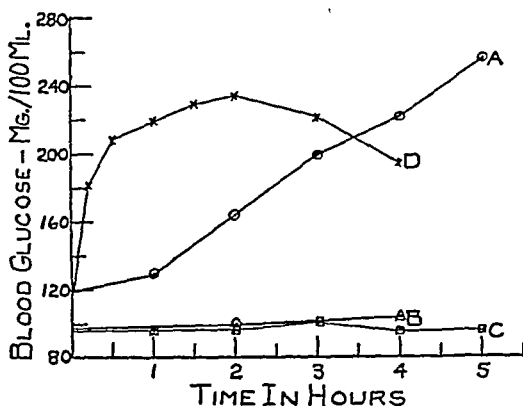


FIG. 1.

The average blood sugar responses of rabbits to the administration of DTB as compared to the response of a rabbit receiving epinephrine. Curve A represents the response to the administration of DTB at a level of 100 mg. per kilogram body weight; Curve B at a level of 40 mg., and, Curve C at a level of 7 mg. per kilogram. Curve D represents the response to epinephrine (0.5 ml. of a 1:1000 solution).

The average curves represented in Figure 1 summarize the results of glucose determinations on rabbits starved for 3 days and then treated with DTB. It may be seen that the administration of the compound in amounts of 7 and 40 mg. per kilogram produced no change in the blood glucose concentration (curves B and C). However, when the DTB was given in a single injection of 100 mg. per kilogram there was an immediate and steady rise in the blood glucose to a peak of 256 mg. per 100 ml. at the end of 5 hours (curve A). It may be said that the glycemic changes encountered in these starved animals were not significantly different from those observed in animals maintained on a normal diet preliminary to the injection of DTB.

Curve D shows the blood glucose response to the intravenous administration of 0.5 ml. of a 1:1000 solution of epinephrine in a rabbit which had been starved for 3 days. It is interesting to observe that an animal, presumably depleted of liver glycogen by starvation, responds to epinephrine with a significant and prolonged rise in the blood sugar. This observation, therefore, does not preclude

the possibility that the blood glucose response to DTB is mediated in some way through epinephrine.

In passing, it may be noted that the rabbits receiving a single injection of DTB at a level of 100 mg. per kilogram of body weight exhibited a flaccid paralysis within several hours after administration of the compound, became cyanotic, and usually continued in this state for a period of eight to seventy-two hours until death intervened.

Effect of DTB on Glucose Utilization. In order to determine the effect of DTB on the utilization of glucose, intravenous glucose tolerance tests were performed on rabbits para-

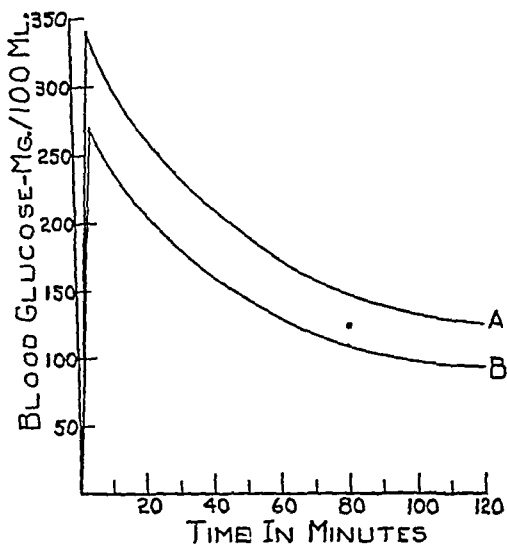


FIG. 2

The average response of DTB-paralyzed rabbits and their pair-fed controls to intravenous administration of glucose. Curve A represents the results obtained with rabbits paralyzed by the daily administration of 7 mg. per kilogram of DTB, while Curve B represents the average results obtained with pair-fed controls.

lyzed by the daily administration of 7 mg. of DTB per kilogram of body weight as an aqueous solution. For the purpose of the tests 0.5 gm. of glucose per kilogram, as a 20% solution, was injected into the marginal ear vein and blood sugar determinations were made every 5 minutes for a period of 2 hours.

In Figure 2 the average results of a number of such experiments are compared with those obtained with pair-fed controls tested similarly. It is apparent that while the hyperglycemic levels produced in the rabbits paralyzed by DTB were higher, that on the average no significant change in the rate of glucose utilization was encountered; and this was true for the individual cases as well.

Although all of the DTB-treated animals on which glucose tolerance tests were performed exhibited a marked paralysis, the fasting blood glucose, in most cases, was normal or only slightly elevated. The above findings lead to the con-

clusion that the rate with which injected glucose is removed from the blood, as measured by the intravenous glucose tolerance test, is the same in rabbits receiving 7 mg. DTB per kilogram body weight as in the control animals. However, since these observations were made on paralyzed animals with an approximately normal fasting blood glucose level, the results are inconclusive as to the situation existing in animals showing abnormalities in carbohydrate metabolism, as indicated by the elevated blood glucose encountered in animals receiving 100 mg. DTB per kilogram. Nevertheless these findings indicate that the effects of DTB on carbohydrate metabolism and on muscular tone are not mutually dependent.

Effect of Insulin and Ergotamine on DTB Hyperglycemia. Both insulin (1 unit per kilogram) and ergotamine (2 ml. of a 1-10,000 solution) given by vein, were found to be antagonistic to the hyperglycemia produced by the administration of DTB in a dosage of 100 mg. per kilogram. Thus, in the presence of hyperglycemia following the injection of DTB, an immediate and marked decline in the blood glucose occurred following the administration of either of these substances.

The effects of DTB on the Thyroid, Thymus and Adrenal Glands of Rats. A group of 4 weanling, female white rats were placed on a normal diet into which was incorporated 0.05% DTB; after 5 days of this regime the DTB concentration was decreased to 0.01%, and the animals permitted to feed for 2 more days. At the end of this time the rats were sacrificed and the weights of their thyroid, thymus, and adrenal glands were determined. Concurrently 4 other weanling rats were maintained for 7 days on the normal diet containing no DTB; at the end of this time these too were sacrificed and their gland weights determined.

Table III summarizes the data obtained in this experiment and the following conclusions may be drawn: (1) On the basis of the *ratio* of thyroid to body weight, DTB was goitrogenic; (2) on any basis, there was marked involution of the thymus gland; (3) on the basis of the ratio of the weight of the adrenal glands to total body weight it may be said that a relative enlargement of the adrenals occurred. In evaluating the significance of these results it must be pointed out that the DTB-treated rats ate very little food, and these glandular changes, typical responses to stress, may be partly in response to the alarming stimulus of inanition. Further, the hyperplasia encountered in the thyroid glands, as evidenced microscopically, was minimal; and many of the glands showed degenerative changes.

The organs of these animals, and it may be added the organs of numerous rabbits and of several dogs receiving DTB, were kindly examined microscopically by Dr. William E. Ehrich who reported finding the pathological stigmata which have come to be associated with reaction to alarming stimuli.

DISCUSSION. The results of studies on the chemical composition of skeletal muscle of rats and rabbits paralyzed with DTB demonstrate that this paralysis is unaccompanied by any significant changes in the water and electrolyte content of skeletal muscle. This observation lends support to the finding of Astwood, Hughes, et al. (2) that DTB paralysis does not involve any disturbance in the muscles themselves.

The changes in the blood glucose produced by the administration of DTB (100 mg. per kilogram) which are reported in this communication are similar to those found by DuBois and co-workers (3, 4) employing thiourea and a number of its derivatives. This suggests that the influence on carbohydrate metabolism of compounds in this general class is similar. These changes were also found to be comparable to those encountered in animals receiving epinephrine. This observation coupled with the finding that insulin and ergotamine exert an antagonistic response to the DTB-hyperglycemia, as do these substances to epinephrine

TABLE III

The effect of DTB on the thyroid, thymus, and adrenal glands of weanling white rats

RAT NO.	INITIAL BODY WEIGHT	FINAL BODY WEIGHT	THYROID WEIGHT	THYMUS WEIGHT	ADRENAL WEIGHT
Control Group					
	gms.	gms.	gms.	gms.	gms.
1	39	60	0.0086	0.2430	0.0145
2	39	56	0.0072	0.2361	0.0145
3	33	54	0.0078	0.2371	0.0122
4	35	60	0.0073	0.2660	0.0140
Average.....	36.5	57.5	0.0077	0.2456	0.0138
DTB Group					
5	35	31	0.0042	0.0319	0.0127
6	34	31	0.0048	0.0264	0.0108
7	36	28	0.0097	0.0206	0.0150
8	34	31	0.0071	0.0210	0.0124
Average.....	34.8	30.3	0.0065	0.0250	0.0127

hyperglycemia, suggests that the effect of DTB on carbohydrate metabolism is mediated through epinephrine.

SUMMARY

1. The administration of DTB to rabbits in amounts of 7, 40, and 100 mg. per kilogram body weight (and to rats in an amount of 0.002% in the drinking water as observed by Astwood, et al.) produced a flaccid and fatal paralysis in from 1 to 12 days, depending on the administered dose.

2. The paralysis produced by the daily administration of 7 mg. DTB per kilogram to rabbits and as a 0.002% solution in the drinking water of rats was unaccompanied by any significant changes in the water, chloride, sodium, potassium, phosphorus, and creatine content of skeletal muscle, and in the few cases studied, in the plasma CO₂ content, calcium, sodium, and potassium levels.

3. The continued administration of DTB to rabbits in an amount of 7 and 40 mg. per kilogram occasionally produced a slight elevation in the blood glucose. When administered at a dosage of 7 mg. per kilogram, DTB had no apparent

effect on the rate of removal of injected glucose from the blood, as measured by the intravenous glucose tolerance test.

4. The administration of a single dose of DTB to rabbits in an amount of 100 mg. per kilogram produced an immediate and profound increase in the blood glucose level which was maintained over a period of 5 hours.

The effects on carbohydrate metabolism and the paralysis accompanying the administration of DTB were not apparently mutually dependent.

5. The effects on carbohydrate metabolism of DTB may be non-specific effects mediated by epinephrine, particularly in view of the fact that the specific glandular changes associated with alarming stimuli have been observed in DTB-treated animals.

BIBLIOGRAPHY

1. ASTWOOD, E. B.: *This Journal*, **78**: 79, 1943.
2. ASTWOOD, E. B., HUGHES, A. M., LUBIN, M., VANDERLAAN, W. P., AND ADAMS, R. D.: *Science*, **102**: 196, 1945.
3. DuBois, K. P., HOLM, L. W., AND DOYLE, W. L.: *Proc. Soc. Exper. Biol. Med.*; **61**: 102, 1946.
4. DuBois, K. P., HERMANN, R. G., AND ERWAY, W. F.: *This Journal*, **89**: 186, 1947.
5. MUNTWYLER, E., MELLORS, R. C., MAUTZ, F. R., AND MANGUN, G. H.: *J. Biol. Chem.*, **134**: 367, 1940.
6. LOWRY, O. H. AND HASTINGS, A. B.: *J. Biol. Chem.*, **143**: 257, 1942.
7. JEGHERS, H. J. AND MYERS, V. C.: *J. Lab. Clin. Med.*, **15**: 982, 1930.

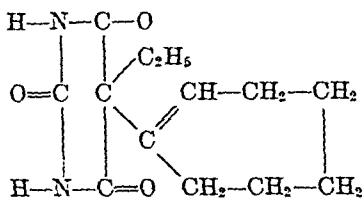
A TOXICOLOGIC AND PHARMACOLOGIC INVESTIGATION OF
CYCLOHEPTENYLETHYL BARBITURIC ACID (MEDOMIN)¹

WILLIAM A. HALBEISEN, CHARLES M. GRUBER, JR. AND
CHARLES M. GRUBER

*Departments of Pharmacology and Medicine, Jefferson Medical College,
Philadelphia, Pennsylvania*

Received for publication January 15, 1948

As far as we are aware, no thorough toxicologic and pharmacologic investigations have been carried out on Cycloheptenylethyl barbituric acid (Medomin).



Fritzsche (1) gives the minimal effective dose for rats as 20 to 30 mgm., and the lethal dose between 800 to 1,200 mgm. a ratio of 1:40. He found the therapeutic index to be 1:3 as compared with 1:2 for phanodorn. However, no experimental data are presented in his report nor does he indicate the route of administration of the drugs. In cats given equal doses of cycloheptenylethyl barbituric acid and phanodorn he found no difference between the two barbiturates in the speed of inducing sleep, and in the depth of hypnosis, but he did find the duration of action to be longer for cycloheptenylethyl barbituric acid. Only after the administration of high toxic doses were changes in the circulatory and respiratory systems observable. Hypnotic and narcotic doses had no influence upon the rate and force of the heart beat, upon the blood pressure and upon respiration.

Fritzche was the first to study the relative hypnotic actions of cycloheptenylethyl barbituric acid and phanodorn in patients suffering from insomnia. In these, the two barbituric acid derivatives were found to be approximately equal in their sleep producing properties. Unlike most long acting barbiturates no "hangover" was observed after the patient awoke from medomin hypnosis. Grote (2) studied the effects of cycloheptenylethyl barbituric acid on eleven human subjects. He observed that 0.2 Gm. given orally produced sleep of 5 to 6 hours duration in cases of insomnia due to surgery, arteriosclerosis, cardiac disease and mental diseases.

Since thorough investigations on the toxicology and pharmacology of the above barbiturate have not been reported and since it is being used clinically in Europe we believe such investigations are in order.

¹ This research was made possible through a grant by Geigy Company, Inc.

METHOD. The toxicologic studies were carried out on 372 white mice weighing between 17 and 28 Gm. having an average weight of 24 Gm., 100 albino rats weighing between 127 and 130 Gm. having an average weight of 128 Gm., 145 rabbits weighing between 1.7 and 2.6 kgm. having an average weight of 2.2 kgm., and 29 mongrel dogs weighing between 7 and 19 kgm.

In mice and rats the experiments were done on groups of 15 to 20 animals and usually the animals were selected according to weight. In rabbits, groups of 5 to 18 animals were used. Thirty or more animals were used for each dose in determining the LD_{50} . In the mice and rats the injections of the drug were made intraperitoneally in 1 and 2 per cent solutions respectively. No distinction was made between sexes although the male sex was predominant. A 5 per cent solution of cycloheptenylethyl barbituric acid was injected into the lateral ear vein of rabbits and into the saphena para vein of dogs. In all of the experiments the drug was weighed as cycloheptenylethyl barbituric acid and a 10 per cent solution of sodium hydroxide (approximately 8 cc./5 Gm.) was added to the drug in a volumetric flask until all of the drug was changed to its sodium salt and a clear solution resulted.

Mongrel dogs weighing between 4 and 18 kgm. were used in those experiments in which the blood pressure and respiration were studied. The animals were first anesthetized with ether by inhalation. A median neck incision was then made and the animal tracheotomized, the ether being continued with the use of an ether bottle. Ether was discontinued upon the injection of the barbiturate. The blood pressure was registered from the right carotid artery by means of a mercury manometer. Heparin was used in the system as the anticoagulant. The time marker which indicated intervals of 20 seconds was placed at the atmospheric pressure line of the manometer. The respirations were recorded upon the kymograph surface by placing about the thorax a pneumograph connected with a Marey tambour. Respiration and blood pressure were recorded simultaneously in most of the experiments. In some experiments the changes in the volume of the spleen, kidney, intestine or limb were also studied. A plethysmograph connected to a modified Brodie bellows was used. In those experiments in which the effect of the drug was studied on the cardiac vagus nerve the right vagus nerve was isolated, sectioned and its distal end stimulated with platinum electrodes connected to the secondary coil of a "Harvard" inductorium.

Tolerance studies were made on rabbits and dogs. In this study the criterion was the same as that employed by Fitch and Tatum (3). The times were recorded at which the animals went to sleep and at which they could first raise their heads, sit upright, maintain that position and hop or walk about when disturbed (4). Sixty-eight rabbits weighing between 2.1 and 3.9 kgm. and 7 dogs weighing between 7 and 10 kgm. were used in these experiments. In the dogs a 10 per cent solution of cycloheptenylethyl barbituric acid (50 mgm./kgm.) was given intravenously through the saphena para vein every other day for a total of three injections and in rabbits a 5 per cent solution (50 mgm./kgm.) was injected into the lateral ear vein daily for four or five days. On the fifth or sixth day, 19 mgm./kgm. of pentobarbital sodium were administered. In another series the same dose of pentobarbital sodium was given daily for four days and on the fifth day cycloheptenylethyl barbituric acid (50 mgm./kgm.) was given intravenously.

In determining the minimal hypnotic dose rabbits were used. The criterion used by Werner, Pratt, and Tatum (5) was followed. "The M. H. D. is that amount of drug in milligrams per kilogram which caused 50 per cent or more of the animals receiving that dose to lie on their sides with head down."

RESULTS. In white mice, 250, 280, and 300 mgm./kgm. of cycloheptenylethyl barbituric acid injected intraperitoneally was observed to kill 12, 45 and 75 per cent respectively of the animals. The LD_{50} was estimated to be 284 mgm./kgm. In rats, 200, 220 and 250 mgm. per kgm. injected intraperitoneally killed 46.6, 50 and 57.5 per cent respectively of the animals. The LD_{50} was estimated to be 220 mgm./kgm.

In rabbits the injections of cycloheptenylethyl barbituric acid were made at a uniform rate (1.2 cc. per minute). At this rate of injection 101, 110 and 120 mgm. per kgm. respectively killed 20, 30 and 52 per cent of the animals injected. The intravenous LD_{50} was found to be 119 mgm. per kgm.

Ninety mgm./kgm. killed none of the dogs, 100 mgm./kgm. killed 33 per cent and 110 mgm./kgm. killed 71 per cent of the animals. The LD_{50} was estimated to be 105 mgm./kgm.

Tolerance. That rabbits and dogs develop a tolerance to barbiturates has been demonstrated on numerous occasions (6). That cycloheptenylethyl barbituric acid when given in repeated doses is no exception can be seen in table I. In the 7 dogs, the average sleeping time following the third administration of 50 mgm./kgm. of the drug was 43 per cent shorter than that following the first. After repeated injections of the drug in groups of 10, 15 and 18 rabbits, the average sleeping time was shortened by 40, 32 and 57 per cent respectively. Since at the time the final test, the ability to hop about when nudged, was made the animals were still definitely depressed, the actual periods of cycloheptenylethyl barbituric acid depression are, of course, much longer than these listed. All of the animals were watched continuously from the time of the injection until they were considered awake. Our experimental animals responded to this barbiturate in much the same way as they did to other barbiturates. The duration of action varied with the animal employed and with the dose injected. Large animals appeared to be more depressed than smaller ones of the same species when the same dose per kgm. was administered. Within limits the larger the dose administered the longer the depression. The average sleeping time was 44 minutes for those rabbits which received 50 mgm./kgm., 63 minutes for those which received 75 mgm./kgm. and 163 minutes for those animals which survived 101 mgm./kgm. Higher dosages had no further effects on the average sleeping times of the survivals.

Although repeated injections had an effect upon the sleeping times of animals it appears to have had no effect upon the toxic effects of the drug. Tolerant animals when given 101 mgm./kgm. slept only 92 minutes as compared to the 163 minutes of non-tolerant animals, nevertheless the mortality rate remained the same for the two groups of animals. With this as with other barbiturates dogs appear to be more susceptible than smaller animals. In dogs 50 mgm./kgm. produced hypnosis lasting for five hours and eleven minutes, 90 mgm./kgm. caused hypnosis of eighteen hours duration and those animals which survived 100 mgm./kgm. slept between twenty and twenty-eight hours.

Cross Tolerance. That dogs, rabbits and rats made tolerant to one barbiturate will very likely show some tolerance to all other barbiturates has been demonstrated before (6). That tolerance to cycloheptenylethyl barbituric acid also influences the sleeping time induced in rabbits by pentobarbital sodium can be seen in table I. After the animals were made tolerant to cycloheptenylethyl barbituric acid, pentobarbital sodium was injected intravenously and it produced a hypnosis of 41 and 39 minutes in the first two groups of rabbits studied (see table I). One week later the same dose of pentobarbital sodium was again injected in these same animals. This time the two groups of animals slept 50

and 52 minutes respectively. The animals were then given daily injections of pentobarbital sodium (19 mgm./kgm.) for four days and on the fifth day cycloheptenylethyl barbituric acid (50 mgm./kgm.) was injected intravenously. Although these two groups of animals had slept on the average of 40 and 50 minutes on the control injection of cycloheptenylethyl barbiturate, after the four injections of pentobarbital these same animals slept for only 19 and 25 minutes, a decrease of 53 and 47 per cent respectively. Oddly enough, the animals receiving daily injections of cycloheptenylethyl barbiturate developed less tolerance to pentobarbital sodium (20 and 25 per cent reduction in sleeping time) than they did to cycloheptenylethyl barbiturate after a series of injections of pentobarbital sodium.

Thirty mgm./kgm. caused 50 per cent or more of the rabbits receiving that dose to lie on their sides with heads down. This dose was considered the minimal

TABLE I

The effects of repeated administrations of cycloheptenylethyl barbiturate and of pentobarbital sodium on the sleeping time in rabbits and dogs

ANIMAL	NUMBER OF ANIMALS	AVERAGE WEIGHT	AVERAGE DURATION OF HYPNOSIS IN MINUTES FOLLOWING DAILY INJECTIONS IN RABBITS AND EVERY OTHER DAY IN DOGS						DECREASE IN SLEEPING TIME IN PER CENT
			1	2	3	4	5	6	
Rabbit*	10	2.3	40	31	25	23	24	41*	40
	15	3.2	47	37	33	31	32	39*	32
	18	2.2	66	45	38				57
Rabbit†	10	2.9	50	38	35	25	19†		50
	15	3.3	52	40	36	31	25†		40
Dog*	7	8.6	292	219	168				43

* Cycloheptenylethyl barbiturate (50 mgm./kgm.).

† Pentobarbital sodium (19 mgm./kgm.).

effective dose. The therapeutic coefficient of cycloheptenylethyl barbiturate is, therefore, calculated to be $\frac{M. L. H.}{M. H. D.} = \frac{119}{30} = 3.9$. This figure is slightly higher than that of phenobarbital sodium (3.3) but lower than that for pentobarbital sodium (4.5).

Respiration and Blood Pressure. In the dogs used in studying the effect of cycloheptenylethyl barbiturate on respiration, blood pressure and organ volume, the drug was injected 27 times in doses of 10, 20, and 25 mgm./kgm.

Within limits the changes in blood pressure are directly proportional to the amount of the drug injected and the rate at which it is injected. Like other barbiturates (7), cycloheptenylethyl even in a small dose causes a fall in blood pressure. If the dose is large, or if small but rapidly injected, there is a rapid fall in blood pressure which requires several minutes to return to its original level. In our experiments 10 mgm./kgm. caused an average fall in blood pressure of 35 per cent, whereas, 20 and 25 mgm./kgm. caused average decreases of 50 and 51 per cent respectively. Following each injection of cycloheptenylethyl

barbiturate the arterial blood pressure gradually returned toward the control level. In figure 1, at M, 25 mgm. per kgm. of the drug were injected intravenously in a 5 kgm. dog. A rapid fall in blood pressure from 130 to 60 mm. Hg occurred.

In those animals in which the blood pressure and rate and depth of respiration were being recorded, marked acceleration of the heart was observed during the injection at the same time the blood pressure was falling. In three trained dogs studied with an electrocardiograph the average heart rate increased from 143 to 205 beats per minute immediately after the injection of 20 mgm./kgm. of the barbiturate. In these the heart rate returned to the control level within 4

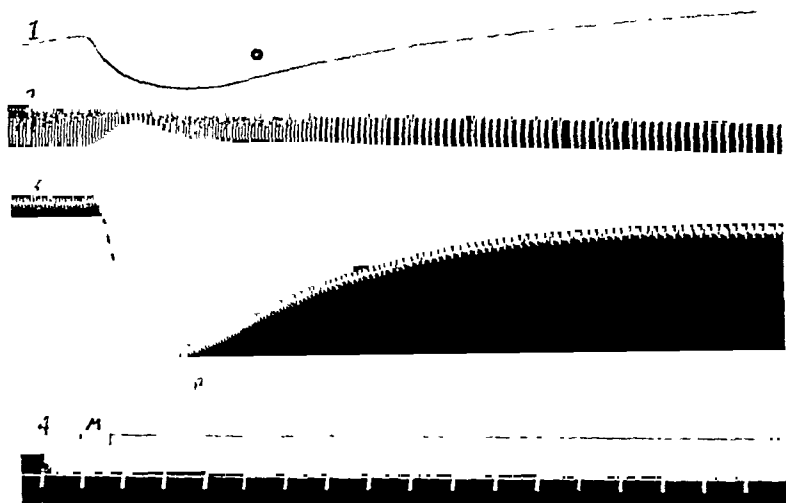


FIG. 1. DOG 5 KGm. ETHER ANESTHESIA

1. Plethysmograph record of spleen. 2. Respiration, up stroke expiration. 3. Arterial blood pressure with Hg manometer. 4. At M, time of intravenous injection of 25 mgm./kgm. of cycloheptenylethyl barbituric acid. 5. Time in intervals of 20 seconds and zero blood pressure.

minutes. No changes in the P-R interval and in the height of the deflections of the string were noted. However, when 50 mgm./kgm. of cycloheptenylethyl barbiturate were injected intravenously the average heart rate during and immediately following the injection was 257 beats per minute requiring 20 minutes to return to the control rate. These animals also slept for over 5 hours.

This increase in heart rate we believe is partly due to a compensatory phenomenon i.e. a reflex from the carotid and aortic sinuses due to the fall in blood pressure and partly due to depression of the cardiac vagus nerves. Experiments were performed on dogs in which the distal end of the cut right vagus nerve was stimulated while the animals were under ether anesthesia, again after the anesthesia was allowed to become very light and finally after the intravenous injection

tion of 50 mgm./kgm. of cycloheptenylethyl barbiturate. In figure 2 can be seen the mildly depressant effect of the barbiturate on the cardiac response to vagus nerve stimulation. While the animal was under deep ether anesthesia the cut right vagus nerve was stimulated, at 1, and a fall of 20 per cent in blood pressure occurred, whereas at 4 when the animal was under extremely light anesthesia a similar stimulation of the nerve caused a 70 per cent drop in blood pressure. Fifty mgm./kgm. of cycloheptenylethyl barbiturate were injected intravenously at B. The vagus nerve was again stimulated, at 5, and at this time a fall of 40 per cent in the blood pressure occurred during the period of excitation of the nerve, however, fifteen minutes after the injection, at 8, the response of the heart to excitation of the vagus nerve was the same as it had been when the animal was under very light ether anesthesia (at 4). At C, 20 mgm./kgm.

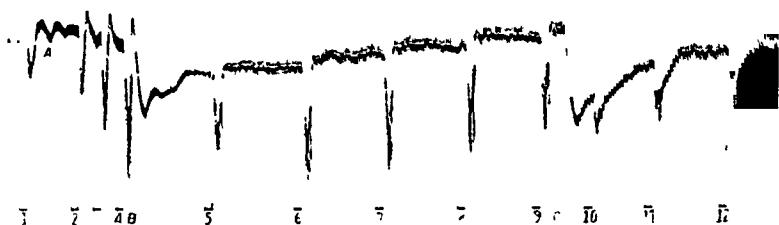


FIG. 2. DOG 11 KGm. ETHER ANESTHESIA USED DURING OPERATION

Top record is that of the arterial blood pressure with Hg manometer. Bottom record indicates the time in intervals of 20 seconds and zero blood pressure. Middle record indicates the time and duration of stimulation of the peripheral end of the cut right vagus nerve. 1. Stimulation of the vagus while the animal was under deep ether anesthesia. At A, the ether was discontinued and the animal allowed to go into light anesthesia. The vagus nerve was again stimulated at 2, 3 and 4 with the same current as that used in 1. At B, 50 mgm./kgm. of cycloheptenylethyl barbiturate was injected intravenously. The vagus nerve was stimulated as before at 5, 6, 7, 8 and 9. Amytal sodium 20 mgm./kgm. was injected intravenously, at C, and subsequently, the vagus nerve was stimulated as before, at 10, 11 and 12.

of amytal sodium were injected intravenously. Following this injection a fall of only 20 per cent in the blood pressure was produced by excitation of the vagus nerve at 10. In the seven dogs studied cycloheptenylethyl barbiturate had less influence on the response of the heart to vagus nerve stimulation than did deep ether anesthesia, amytal sodium or seconal sodium. The mild depression produced we believe, however, is adequate to account in part for the acceleration of the heart immediately following the injection of the drug.

Cycloheptenylethyl barbiturate like all other barbiturates studied depresses both the rate and the depth of respiration. Large doses injected rapidly caused complete cessation of respiration in expiration although the heart continues to beat rhythmically for some minutes. During the sudden fall in blood pressure an acceleration of respiration frequently occurs (figure 1). This we believe to be a reflex from the carotid and aortic bodies due to anoxia. The rate of respiration of the animal used in figure 1 was 33 respirations per minute, before the in-

jection of the drug. After the injection and at the time of maximum fall in blood pressure the respiratory rate was 48 per minute and as the blood pressure recovered the respiratory rate gradually decreased to 15 respirations per minute. Another injection slowed respiration to 12 respirations per minute and following a fourth injection, respiration ceased although the heart continued to beat for many minutes. After respiration had ceased, artificial respiration or the intravenous injection of epinephrine frequently brought about recovery of respiration even when doses as large as 100 mgm./kgm. of cycloheptenylethyl barbiturate had been administered.

The changes in the volumes of the spleen, kidney, intestine and limb, following the intravenous administration of the drug were not constant. In most instances there was a decrease in the volume of the plethysmographed organ which we believe was passive in character, due to the sudden fall in systemic blood pressure. This change can be seen in figure 1. When the fall in blood pressure was not too extensive the volume of these organs increased. In dogs visible swelling and flushing of the skin occurred during and immediately following the injection of the compound.

Cycloheptenylethyl barbituric acid, like other intermediate acting barbiturates, is apparently destroyed in the body. Only one oxidation product "Cycloheptenylethyl barbituric acid" has thus far been isolated from the urine of rabbits and human beings (8). In the experimental animals as much as 2.3 to 2.8 per cent of the total amount of the drug administered subcutaneously was excreted as this oxidation product, but in man, less than one per cent was found in the urine. This oxidation product possesses no narcotic action and is only 1/20 as toxic as the parent compound for mice. No free cycloheptenylethyl barbituric acid was found in the urine. That this barbiturate is destroyed in the body has been confirmed by our experiments on nephrectomized rabbits. The average sleeping time for 17 nephrectomized rabbits following the intravenous injection of 50 mgm./kgm. of cycloheptenylethyl barbiturate was 61 minutes as compared to 65.8 minutes for the control animals. Likewise in our experiments on a few dogs no notable difference in sleeping times was seen in normal and nephrectomized animals.

SUMMARY

1. The LD_{50} of cycloheptenylethyl barbituric acid injected intraperitoneally is 284 mgm./kgm. for white mice, and 220 mgm./kgm. for albino rats. When the drug is injected intravenously the LD_{50} is 119 mgm./kgm. for rabbits, and 105 mgm./kgm. for dogs.
2. Using the duration of induced sleep as the criterion, both rabbits and dogs develop a tolerance for cycloheptenylethyl barbituric acid.
3. Animals rendered tolerant to this barbiturate are also tolerant to pentobarbital sodium and vice versa.
4. Large doses of cycloheptenylethyl barbituric acid rapidly given intravenously produce a sudden fall in arterial blood pressure. The extent of fall is directly proportional to the amount given and to the speed of intravenous ad-

ministration. An increased heart rate occurs during the fall and persists for some minutes after the blood pressure has returned to the control level.

5. Large intravenous doses of cycloheptenylethyl barbiturate given rapidly produce marked slowing, and may cause permanent cessation, of respiration in expiration. The respiratory mechanism fails before the heart.

6. In the experiments in which the fall in blood pressure is not extensive there appears to be a dilation of the vessels of the spleen, intestine, kidney and limb. In all of the experiments in which the fall in blood pressure is sudden and extensive a decrease in the volume of these organs is observed which we believe to be passive in character. The vessels of the skin dilate.

7. This barbiturate appears to have less depressant effect upon the cardiac vagus nerves than do other intermediate acting barbiturates such as amytal sodium and seconal sodium.

8. Like other intermediate acting barbiturates cycloheptenylethyl barbiturate is destroyed in the body and is excreted as the parent substance only when excessively large doses are given.

REFERENCES

1. FRITZSCHE, R.: *Praxis*, 31: 740, 1942.
2. GROTE, F.: *Schweitz. Med. Wochenschr.*, 72: 1333, 1942.
3. FITCH, R. H., AND TATUM, A. L.: *This Journal*, 44: 325, 1932.
4. EDDY, N. B.: *This Journal*, 33: 43, 1923.
5. WERNER, H. W., PRATT, T. W., AND TATUM, A. L.: *This Journal*, 60: 189, 1937.
6. GRUBER, C. M., AND KEYSER, G. F.: *This Journal*, 86: 186, 1945.
7. GRUBER, C. M., AND BASKETT, R. F.: *This Journal*, 25: 219, 1924; *Jour. Lab. & Clin. Med.*, 10: 630, 1925. GRUBER, C. M.: *This Journal*, 60: 143, 1937. GRUBER, C. M., ELLIS, F. W., AND FREEDMAN, G.: *This Journal*, 81: 254, 1944.
8. FRITZSCHE, R.: *Loc. cit.*; PULVER, R.: *Schweiz. Med. Wochenschr.*, 73: 124, 1943.

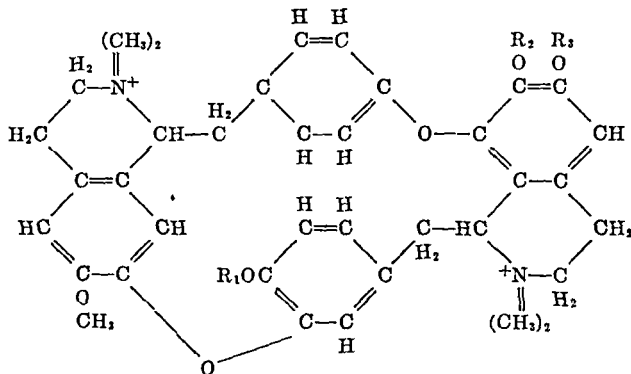
THE CURARIFORM ACTIVITY OF d-N-METHYL-CHONDRODENDRINE AND d-O-METHYL-N-METHYL-CHONDRODENDRINE¹

DAVID F. MARSH, CLARK K. SLEETH, AND ELDON B. TUCKER

Departments of Pharmacology and Medicine, West Virginia University School of Medicine, Morgantown

Received for publication January 19, 1948

King (1) in his elucidation of the structure of d-tubocurarine indicated the close chemical similarity of d-N-methyl-chondrodendrine and established the isomerism of the two dimethyl ethers, d-O-methyltubocurarine and d-O-methyl-N-methyl-chondrodendrine. In spite of the ready availability of d-chondrodendrine² (2) from various *Chondrodendron* species, and the ease with which the simple alkyl derivatives can be prepared (3, 4), apparently no quantitative study of the curariform activity of these compounds has been made. We have compared d-N-methyl-chondrodendrine iodide and d-O-methyl-N-methyl-chondrodendrine iodide³ with d-tubocurarine chloride and d-O-methyltubocurarine iodide.



d-Tubocurarine: $R_1 = H$; of R_2 and R_3 , one is H the other CH_3 . Molecular weight 624.7. Used as the dichloride·pentahydrate, molecular weight 785.7. M. p. 268–269°C.

¹ Part of the material in this paper was presented before the American Society of Pharmacology and Experimental Therapeutics, Federation meetings, Atlantic City, 1948. See Federation Proceedings 7: 243, 1948.

² This alkaloid is also known variously as d-bebeerine, d-chondodendrine, and d-curine.

³ We are grateful to Dr. W. G. Bywater of S. B. Penick & Co., New York, for a generous supply of "Radix pareira brava", a crude drug containing chondrodendrine, and to Dr. D. L. Tabern of Abbott Laboratories, North Chicago, Illinois, for a sample of d-O-methyl-N-methyl-chondrodendrine iodide (labelled "bebeerine dimethylether methiodide") for comparison purposes. After this research was completed, we received a sample of "Curare-Like Substance", a solution of d-O-methyl-N-methyl-chondrodendrine chloride, from Dr. Bywater. With due allowance for the difference in molecular weight of the halide salts, these two compounds were identical in activity with the material that we prepared.

d-O-Methyltubocurarine: R_1 , R_2 , and R_3 are CH_3 . Of the two centers of asymmetry, one is dextrorotatory, the other levorotatory. Molecular weight 652.8. Used as the diiodide trihydrate, molecular weight 960.7. M. p. 257–258°C. (Anhydrous salt, M. p. 268–270°C.).

d-N-Methyl-chondrodendrine: $R_1 = H$; of R_2 and R_3 , one is H the other CH_3 . Molecular weight 624.7. Used as the diiodide, molecular weight 878.6. M. p. 251–253°C.

d-O-Methyl-N-methyl-chondrodendrine: R_1 , R_2 , and R_3 are CH_3 . Both centers of asymmetry are dextrorotatory. Molecular weight 652.8. Used as the diiodide, molecular weight 906.6. M. p. 257–258°C.

EXPERIMENTAL.

Rats. The relative toxicity was first determined in 160 albino male rats (170–250 Grams). Solutions containing 0.10 mgm. curariform ion per cc. were injected intraperitoneally and

TABLE 1

	d-TUBOCURARINE	d-O-METHYLTUBOCURARINE	d-N-METHYLCHONDRODENDRINE	d-O-METHYL-N-METHYLCHONDRODENDRINE
Albino rats				
LD 50.	0.22 (0.27)*	0.022 (0.032)	0.39 (0.55)	0.27 (0.37)
Rabbits				
Head drop 50.	0.10 (0.12)	0.011 (0.016)	0.16 (0.23)	0.08 (0.11)
Holaday Head drop.	0.12 (0.15)	0.014 (0.020)	0.19 (0.27)	0.09 (0.13)
LD 50.	0.28 (0.35)	0.027 (0.040)	0.28 (0.40)	0.17 (0.23)
Cat Gastrocnemius Muscle				
Equivalent Paralysis.	0.04 (0.05)	0.005 (0.007)	0.28 (0.40)	0.04 (0.05)
Man				
Head drop.	0.12 (0.15)	0.020 (0.030)	0.43 (0.60)	0.11 (0.15)

* All doses given in mgm. of curariform ion per kgm. body weight. Dose of equivalent amount of salt used given in parenthesis. The animal data was treated by the method of Miller and Tainter (9). The standard errors of all the rat data lie within 3% of the figures given, within 4% for the rabbit data, within 7% for the cat data.

the lethal doses determined (see Table 1). Four to 10 minutes after receiving a lethal dose, the animals became limp and unable to walk, respiration stopped in an additional 3 to 6 minutes, and finally cardiac activity ceased. The rats did not show any signs that any of these agents had cholinergic activity. They did not sneeze or salivate or evidence chromodachyria or flush as they do with some samples of crude curare.

Rabbits. Solutions containing 0.25 mgm. curariform ion per cc. were injected in fifteen seconds into the marginal ear vein of 36 rabbits (1.6–3.0 kgm.) individually restrained in an enclosed box. The doses producing head drop lasting a minimum of three minutes in half of a group of 8 animals were determined (see Table 1). The experiments were repeated on the same animals after a one week interval. After an additional seven day rest period, the head drop cross-over assay of Holaday, as elaborated by Chase, Schmidt and Bhat-tacharya (5) was performed. By this technique, increments of agent are added at fifteen second intervals. This occurs in all animals. Finally, the dose producing respiratory paralysis was determined by this increment technique. This total dose was injected

in one minute or less in groups of four rabbits, and the series expanded until the dose was found that killed four of eight rabbits

Cats Three hundred mgm of sodium barbital and 1 mgm of atropine sulfate per kgm were administered intraperitoneally 60-90 minutes prior to operation in eight cats (1.8-3.1 kgm.) The femoral and sciatic nerves to one leg were cut. The peripheral end of the cut sciatic nerve was stimulated for one tenth second with 6 volts 60 cycle half wave every

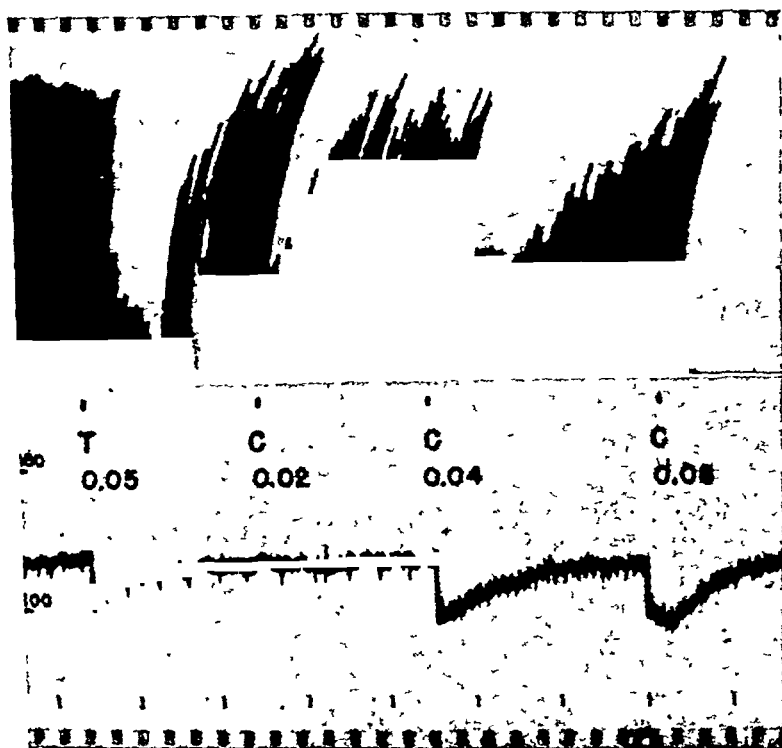


FIG. 1

30 Kgm. male cat. Gastrocnemius contractions, above, and blood pressure, in mm. Hg, below. Time marks at 5 minute intervals

0.05 Mgm d-tubocurarine chloride pentahydrate per kgm given at T

0.02, 0.04, and 0.08 Mgm d O-methyl-N-methyl-chondrodendrine per kgm given at C. The contractions of the gastrocnemius muscle never returned to normal after the last injection

ten seconds by a motor driven interrupter. The contractions of the gastrocnemius muscle were recorded on a soot kymograph with a weighted lever. Carotid blood pressure was recorded with the usual mercury manometer. The cats were mechanically oxygenated.

Fifty micrograms d-tubocurarine chloride pentahydrate per kgm was given intravenously as a reference paralyzing dose. After the muscle returned to normal, various doses of the other agents were given until equivalent paralysis was produced (see Figure 1). The muscle could be partially paralyzed by this procedure for three to eight times before contractility no longer returned to normal.

Man. An 80 kgm. 28 year old white male was placed in a modified Fowler's position on a treatment table (back elevated about 40° from the horizontal, legs about 120° to trunk). A strap was placed around the lower thighs to prevent the subject from slipping off the table. A standardizing dose of d-tubocurarine chloride was administered into an arm vein in a two minute interval and the effects noted. Based on these results, the following dosage procedure was devised. At 48 hour intervals the agents were administered intravenously until severe, apparently maximal, ptosis resulted. An additional one-fourth of this amount was given in the time remaining in the two minute administration period. This total dose produced head drop lasting three to five minutes.

Within thirty seconds following the two minute injection period, the individual evidenced ptosis, nystagmus, dysarthria, and rapidly developing generalized dyskinesia which became maximal in an additional 90 seconds, with inability to elevate the head. Respiration was entirely diaphragmatic. The systolic and diastolic blood pressure each fell less than 10 mm. Hg. There was no change in pupil size nor gross evidence of excessive salivation. The effects disappear rapidly following the administration of doses of any of these agents producing 3 minute head drop. The individual was able to walk steadily within thirty minutes. No neostigmine methylsulfate was administered.

The subject stated that there was no feeling of excitement nor loss of pain sensation or consciousness. There was no nausea nor desire to defecate or urinate during the procedure. The most distressing symptom was the inability to swallow. There was a transient feeling of tingling or flushing in the extremities following the injection. Diplopia was the first subjective indication that an agent was having an effect, and this persisted for as much as two hours after an experiment. There were no noticeable after-effects. The subject did not believe that he would be able to distinguish among these drugs and felt that they were all identical in subjective effect.

Doses of d-tubocurarine chloride that produced head-drop for as long as 18 minutes and intercostal paralysis for 10 minutes did not have any noticeable histamine-like effects in the subject, a known asthmatic, although there was a feeling of tightness of the lower chest which was different from the feeling of asthma or bronchospasm. The effects of these agents are in general agreement with those described for d-tubocurarine by Smith (6).

RESULTS. All of these compounds have the same specific action. They produce skeletal muscular paralysis and have little other direct observable activity in the animal body. The chondrodendrine derivatives differ from the tubocurarine compounds only in their quantitative activity. d-N-Methyl-chondrodendrine is about one-half as active as the isomeric d-tubocurarine in rats and rabbits, although only one-fourth to one-eighth as active in cats and man. Although the d-O-methyl-N-methyl-chondrodendrine is about equipotent with d-tubocurarine, it is only one-sixth to one-eighth as active as its diastereoisomer, d-O-methyltubocurarine. The results of the Holaday type assay for d-tubocurarine and d-O-methyltubocurarine are in good agreement with those quoted by Dutcher (3).

DISCUSSION. Drugs which differ only by internal asymmetry usually differ in quantitative activity, but not in qualitative physiological activity. l-Epinephrine is several times more potent than d-epinephrine, although the type of action observed for both is the same. d-Tubocurarine has one dextrorotatory and one levorotatory center of asymmetry; both centers of asymmetry are dextrorotatory in d-N-methyl-chondrodendrine (7). l-Tubocurarine has the other possible relationship; i.e., the reverse of d-tubocurarine, with one levorotatory and one dextrorotatory center. d-N-methyl-chondrodendrine, which

has one center of asymmetry the same as and one center opposite from the relationship found in d-tubocurarine, has only half the activity of d-tubocurarine. As one might expect, l-tubocurarine, which has both centers with the opposite relationship, has the least activity, being only 1/30 to 1/60 as active as d-tubocurarine (8).

Conversion of d-tubocurarine to the dimethylether, d-O-methyltubocurarine, increases its potency eight to ten times. Similarly, conversion of d-N-methyl-chondrodendrine to d-O-methyl-N-methyl-chondrodendrine produces a material that is six to eight times more potent.

Although the potencies of the chondrodendrine derivatives are less than the tubocurarine compounds, the similar lack of undesirable side effects in man and greater potential availability indicate the need for their clinical investigation.

SUMMARY

d-N-Methyl-chondrodendrine iodide and d-O-methyl-N-methyl-chondrodendrine iodide were compared with d-tubocurarine chloride pentahydrate and d-O-methyltubocurarine iodide trihydrate in rats, rabbits, cats, and man. d-N-Methyl-chondrodendrine (as the ion) is about one-half as paralyzant as the isomeric d-tubocurarine in rats and rabbits, although only one-fourth to one-eighth as active in cats and man. d-O-Methyl-N-methyl-chondrodendrine is about equipotent with d-tubocurarine, but it is only one-sixth to one-eighth as active as its diastereoisomer, d-O-methyltubocurarine. All of these compounds have relatively little effect in intact animals other than lissive action on skeletal muscles.

BIBLIOGRAPHY

1. KING, H.: J. Chem. Soc., 1935: 1381.
2. KING, H.: J. Chem. Soc. 1940: 737.
3. Dutcher, J. D.: J. Am. Chem. Soc., **68**: 419, 1946.
4. BRAZIL, O. V., SEBA, R. A., AND CAMPOS, J. S.: Bol. Inst. Vital Brazil, **5**: 79, 1945.
5. CHASE, H. F., SCHMIDT, J. L., AND BHATTACHARYA, B. K.: THIS JOURNAL, **91**: 236, 1947.
6. SMITH, S. M., BROWN, H. O., TOMAN, J. E. P., AND GOODMAN, L. S.: Anesthesiology, **8**: 1, 1947.
7. KING, H.: J. Chem. Soc., 1937: 1472.
8. KING, H.: J. Chem. Soc., 1947: 936.
9. MILLER, L. C. AND TAINTER, M. L.: Proc. Soc. Exptl. Biol. and Med., **57**: 261, 1944.

PHARMACOLOGICAL PROPERTIES OF SYMPATHOMIMETIC DIAMINES

G. LEHMANN AND LOWELL O. RANDALL

Scientific Department, Hoffmann-La Roche, Inc., Nutley, New Jersey

Received for publication January 28, 1948

The exchange of "isosteric" groups such as methyl, hydroxyl or amino groups often yields compounds with similar pharmacological properties. A typical example as pointed out by Hartung (1) is the close pharmacological similarity of phenylethanolamine and β -phenylpropylamine, which represents an exchange of the hydroxyl by a methyl group. Little is known about the effect produced by the introduction of a second amino group into the side chain and in particular by substituting an amino group for the alcoholic hydroxyl group in alkanolamines such as epinephrine and ephedrine. The reports in the literature concerning the chemistry of phenylalkylenediamines are scanty (2, 3), presumably due to the difficulties encountered in synthesizing this type of compound (4, 5). Since completion of this investigation Funke and Bovet (6) reported on the vasopressor action of a few primary diamines.

Dr. R. Duschinsky and Mr. L. A. Dolan have discovered a general and convenient method (7) allowing the synthesis of a series of such diamines. The pharmacological properties of these diamines* have been studied and compared in some instances with the corresponding monoamines.

Toxicity. Intraperitoneal toxicity was determined in mice and LD₅₀ values were calculated in the usual manner from the log dose—percent mortality curves (Table I). The low toxicity of all diamines is striking. Introduction of one or two phenolic hydroxyl groups is of little influence on toxicity. The primary amines are slightly more toxic than secondary ones. Substitution of cyclohexyl for phenyl in the phenyl-2,3-propanediamines yields compounds with higher toxicity.

In general, replacement of a hydroxy group by the amino group results in a decrease in toxicity. Thus the diamines, 1-(3,4-dihydroxyphenyl)-N²-methylethylenediamine (Nu-1408) and 1-(3-hydroxyphenyl)-N²-methylethylenediamine (Nu-1683), are considerably less toxic than the corresponding monoamines, epinephrine and 'Neosynephrine', respectively. Similarly, the diamines possess lower toxicity than their analogs in which an H-atom replaces the second amino group. As an example, 1-phenyl-1,2-propanediamine (Nu-1318) is one-sixth as toxic as amphetamine.

Signs of sympathetic stimulation such as salivation, exophthalmus and pilomotor reaction are observed in mice after toxic doses of almost all diamines.

The effect on coordinated activity of mice was observed according to the

* All diamines are racemic compounds, if not otherwise mentioned. Wherever both amino groups are attached to assymetric carbon atoms *cis*-configuration is assumed in view of the method of preparation used (7, 8, 9).

method of Gunn and Gurd (10). Signs of central stimulation were evident only with diamines having a propyl side chain. The activity of such diamines as Nu-1272, Nu-1473, Nu-1318 and Nu-1410 is essentially the same as that of ephedrine but is shorter in duration.

CIRCULATION. Pressor potency and duration of action of all compounds was determined on intravenous administration in decapitated cats and in dial-urethane anesthetized cats and dogs. At least three animals were used for each compound. Epinephrine served as reference compound. When tachyphylaxis was apparent, only the first injection was considered for evaluation of pressor potency. All data concerning pressor activity and duration of action relative to epinephrine are presented in Table 1.

Some of the diamines, such as Nu-1408, Nu-1825, Nu-1683, and the d-isomer of the latter, are potent pressor substances. They are, however, one-tenth as active as the corresponding compounds, epinephrine, arterenol and 'Neosynephrine' which possess an alcoholic hydroxyl group. A similar relationship exists between the amino and hydroxy compounds of the phenylpropane series. Nu-1272 and Nu-1318 are one-third as active as ephedrine and 'Propadrine.' There is, however, no definite relationship between the pressor potency of diamines and that of the corresponding compounds in which an H-atom replaces the second amino group. Nu-1408 equals 'Epinine' (11) in pressor activity and Nu-1271 and Nu-1318 are as potent as desoxyephedrine and amphetamine, whereas the diamines corresponding to phenethylamine, 'Paredrine' and 'Tuamine' are less active than the latter.

As to the relationship between structure and pharmacological action among diamines we find that it follows rules established for monoamines (1). Catechol derivatives have greater pressor activity than phenol derivatives and the latter have greater activity than unsubstituted phenyl derivatives. There is little difference in pressor activity between corresponding primary and secondary amines.

According to Funke and Bovet (6) the amino analogs of phenylethanolamine (Nu-1472) and of arterenol (Nu-1825) appear to have equal pressor activity, while we found Nu-1825 almost one hundred times stronger than Nu-1472.

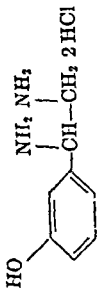
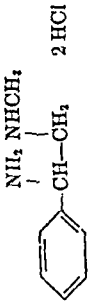

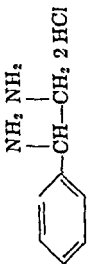
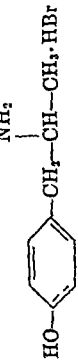
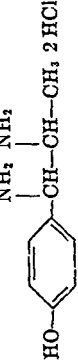
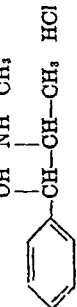
Duration of action is shorter with the diamines of the phenylpropane type than with the corresponding mono-amines. The relationship in this respect is, however, reversed with analogous compounds possessing one or two phenolic hydroxyl groups. The effect of 1-(m-hydroxyphenyl)-N²-methylethylene diamine (Nu-1683) is of considerably longer duration than that of an equi-active dose of 'Neosynephrine.'

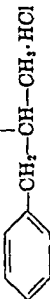
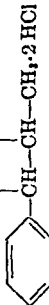
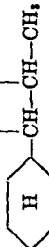


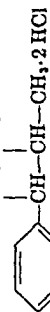
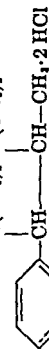
The arylpropanediamines showed marked tachyphylaxis, while the arylethylenediamines and the arylbutanediamines did not.

Nu-1683, like 'Neosynephrine,' is active when injected into the jejunum. In 3 out of 5 dogs in dial-urethane anesthesia, 10 mgm. of Nu-1683 per kgm. produced a pronounced and long-lasting rise in arterial pressure as illustrated in figure 1.

TABLE I
Toxicity and pressor action

COMPOUND	STRUCTURE	TOXICITY I.P. IN MICE LD50 mg./kg.	RELATIVE PRESSOR ACTIVITY				RELATIVE DURATION OF PRESSOR ACTION		
			Dogs (dial)	Cats (dial)	Cats (decap.)		Dogs (dial)	Cats (dial)	Cats (decap.)
Epinephrine.....		13	1	1	1		1	1	1
Nu-1408.....		700	1/20	1/10	1/8-1/10		2	1	1
Nu-1825.....		530	1/25	1/6	1/10		2	1	1
Neosynephrine.....		330	1/20	1/10	1/20		5	2	1
Nu-1683.....		1000	1/200	1/200	1/400		20	0	3

Nu 2013	d isomer of Nu-1683	950	1/125	1/125	1/200	20	7	
Nu 2014	l-isomer of Nu-1683	1400	1/1000	1/1000		10	4	
Nu 1896		700	1/250	1/250	1/200	20	8	4
Nu-1866		760		1/2000			2	
Phenethylamine		200			1/200			3
Nu 1472		600			1/900			4
Paredrine		430			1/100			6-20
Nu-1173		800			1/250			7-11
Ephedrine		325	1/200	1/100	1/125	20	8	4

COMPOUND	STRUCTURE	TOXICITY I.P. IN MICE LD50 mg./kg.	RELATIVE PRESSOR ACTIVITY			RELATIVE DURATION OF PRESSOR ACTION		
			Dogs (dial)	Cats (dial)	Cats (decap.)	Dogs (dial)	Cats (dial)	Cats (decap.)
Desoxyephedrine.....		75			1/500		8-10	
Nu-1272.....		400			1/350		2	
Nu-1410.....		425			1/1000		2	
Propadrine.....		300			1/150		4	
Benzedrine.....		75			1/400		7	
Nu-1318.....		800			1/300		2	
Nu-1665.....		345		1/4000			1	

Nu-1703	$ \begin{array}{c} \text{NH}_2, \text{NH}_2 \\ \quad \\ \text{---CH---CH---} \\ \quad \\ \text{C}_6\text{H}_5 \quad \text{C}_6\text{H}_5 \cdot 2 \text{HCl} \end{array} $	320	1/4000		2	1
Nu-1638	$ \begin{array}{c} \text{OH} \quad \text{NH}_2, \text{NH}_2 \\ \quad \\ \text{---CH---CH---} \\ \quad \\ \text{C}_6\text{H}_5 \quad \text{C}_6\text{H}_5 \cdot 2 \text{HCl} \end{array} $	>800	1/1000		2	
Nu-1613	$ \begin{array}{c} \text{NH}_2, \text{NH}_2 \\ \quad \\ \text{---CH}_2\text{---CH---} \\ \quad \\ \text{C}_6\text{H}_5 \quad \text{C}_6\text{H}_5 \cdot 2 \text{HCl} \end{array} $	>600	1/1000		2	
Nu-1637	$ \begin{array}{c} \text{OH} \quad \text{NH}_2, \text{NH}_2 \\ \quad \\ \text{---CH---CH---} \\ \quad \\ \text{H} \quad \text{C}_6\text{H}_5 \cdot 2 \text{HCl} \end{array} $	1250	1/1000		2	
Nu-1656	$ \begin{array}{c} \text{NH}_2, \text{NH}_2 \\ \quad \\ \text{---CH}_2\text{---CH---} \\ \quad \\ \text{H} \quad \text{C}_6\text{H}_5 \cdot 2 \text{HCl} \end{array} $	320	1/1000		1	
Nu-973	$ \begin{array}{c} \text{H}_2\text{N} \quad \text{NH}_2 \\ \quad \\ \text{---CHOH---CH---CH---} \\ \quad \quad \\ \text{C}_6\text{H}_5 \quad \text{C}_6\text{H}_5 \quad \text{H}_2\text{SO}_4 \end{array} $	820	1/8000			1
Nu-965	$ \begin{array}{c} \text{H}_2\text{N} \quad \text{NH}_2 \\ \quad \\ \text{---CH}_2\text{---CH---CH---} \\ \quad \quad \\ \text{C}_6\text{H}_5 \quad \text{C}_6\text{H}_5 \quad \text{H}_2\text{SO}_4 \cdot \text{H}_2\text{O} \end{array} $	610	1/8000			1
Nu-935	$ \begin{array}{c} \text{NH}_2, \text{NH}_2 \\ \quad \\ \text{---CH}_2\text{---CH---CH---} \\ \quad \\ \text{H} \quad \text{C}_6\text{H}_5 \cdot 2 \text{HCl} \end{array} $	250	1/8000			1

TABLE 1—continued

COMPOUND	STRUCTURE	TOXICITY I.P. IN mg/LD50	RELATIVE PRESSOR ACTIVITY			RELATIVE DURATION OF PRESSOR ACTION		
			Dogs (dial)	Cats (dial)	Cats (decap)	Dogs (dial)	Cats (dial)	Cats (decap)
Tuamine	$\begin{array}{c} \text{NH}_2 \\ \\ \text{CH}_3(\text{CH}_2)_6\text{CH}-\text{CH}_3 \cdot \text{HCl} \end{array}$	75			1/125			3
Nu-1388	$\begin{array}{c} \text{NH}_2 \text{ NH}_2 \\ \quad \\ \text{CH}_3-\text{CH}-\text{CH}-(\text{CH}_2)_3\text{CH}_3 \cdot 2 \text{HCl} \end{array}$	550			1/3000			1
Nu-2169	$\begin{array}{c} \text{NH}_2 \text{ NH}_2 \\ \quad \\ \text{CH}_3(\text{CH}_2)_4\text{CH}-\text{CH}_2 \cdot \text{HCl} \end{array}$	575			Fall			
Nu-1409	$\begin{array}{c} \text{NH}_2 \text{ NH}_2 \\ \quad \\ \text{CH}_3-\text{CH}-\text{CH}-(\text{CH}_2)_3\text{CH}_3 \cdot 2 \text{HCl} \end{array}$	250			Fall			
Nu-1813	$\begin{array}{c} \text{NH}_2 \text{ NH}_2 \\ \quad \\ \text{H}_3\text{C}-\text{CH}-\text{CH}-(\text{CH}_2)_{10}\text{CH}_3 \cdot 2 \text{HCl} \end{array}$	110			Fall			

The qualitative action of Nu-1408 on arterial pressure was further analyzed in comparison with epinephrine. Nu-1408 produced a pure pressor effect; the depressor component usually observed with epinephrine was absent (figure 2). In cats, the pressor action of Nu-1408 was abolished but not reversed by ergotamine. In dogs, however, the action of Nu-1408 was reversed after the intravenous injection of 5 mgm. of yohimbine per kgm. In cats, cocaine potentiated the pressor response to Nu-1408 to a greater degree than that to epinephrine.

In the course of studies on stability an interesting phenomenon was observed. The pressor activity of one per cent solutions of Nu-1408 in the presence of 0.1 per cent sodium bisulfite, although unaltered by heat sterilization and by "ageing" for 500 hours at 45° C, was enhanced three-fold after exposure to ultraviolet light for 16 hours. The irradiated solution had, as figure 2 demonstrates, a biphasic action on arterial pressure in the etherized cat and gave a depressor effect after ergotamine. This suggests that epinephrine had been

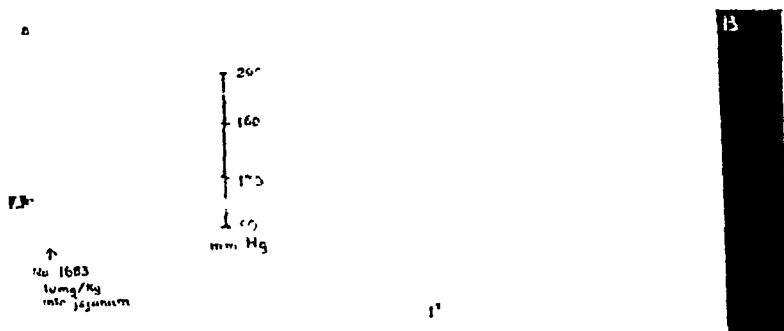


FIG. 1. EFFECT OF 1-(M-HYDROXYPHENYL)-N³-METHYL ETHYLENEDIAMINE DIHYDROCHLORIDE(Nu-1683) ON INTRA-INTESTINAL INJECTION

Dog 9.6 Kg., dial-urethane.

From the top down, carotid arterial pressure, time in minutes. At arrow, Nu-1683, 10 mgm./kgm. into jejunum. Between A and B 30 minutes.

formed from Nu-1408 on irradiation with ultraviolet light. In the absence of sodium bisulfite, solutions of Nu-1408 turned pink on standing. The oxidation seemed to proceed more slowly than with solutions of epinephrine.

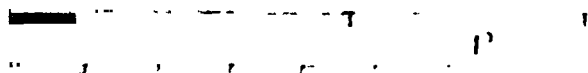
The action of some diamines has been tested on the isolated perfused rabbit's heart. When compared to epinephrine, the compounds show a positive inotropic action which is weaker than to be expected from the pressor ratio.

ISOLATED SMOOTH MUSCLE. Spontaneous motility of the isolated rabbit's intestine suspended in Locke's solution is inhibited by most of the diamines, but all are considerably weaker than the corresponding mono-amines.

Bronchodilator potency relative to epinephrine was determined by the method of Castillo and deBeer (12). At least three preparations have been used for each compound. The results are compiled in table 2 and a typical record is shown in figure 3. Nu-1408, is about 1/20 as active as the corresponding mono-amine, epinephrine, but ten times more potent than the primary diamine, Nu-1825. A similar potency relationship exists between Nu-1683 and Nu-1896.

A

Nu-1408 .03 mg/kg Epi .0025 mg/kg Nu-1408 .01 mg/kg



B

A.P.

120
80
mm Hg

Epi
.0025 mg/kg

Nu-1408
.03 mg/kg

Nu-1408 UV
.01 mg/kg

FIG. 2. ACTION OF 1-(3,4-DIHYDROXYPHENYL)-N²-METHYL ETHYLENEDIAMINE DIHYDROCHLORIDE (Nu-1408) ON ARTERIAL PRESSURE

Cat 3.5 Kg., ether anesthesia.

From the top down, carotid arterial pressure, signal of injection, time in minutes.

From left to right at: A. Nu-1408, 0.03 mgm./kg.; epinephrine, 0.0025 mgm./kg.; Nu-1408 (u.v.)*, 0.01 mgm./kg. B. after egotamine tartrate, 1 mgm./kg; epinephrine 0.0025 mgm./kg.; Nu-1408, 0.03 mgm./kg.; Nu-1408 (u.v.)*, 0.01 mgm./kg. All injections through cannula into femoral vein.

* u.v. = irradiated with ultraviolet light.

OXIDATION BY AMINE-OXIDASE. The susceptibility to amine-oxidase (guinea pig's liver brei) of some of these diamines has been studied, using tyramine as standard, by measuring the rate of oxidation in the Warburg apparatus. The

A

↑
epi
 5×10^{-3}

↑
epi
 10^{-3}

↑
epi
 2×10^{-3}

B

↑
Nu-1408
 10^{-3}

↑
Nu-1408
 2×10^{-3}

↑
Nu-1408
 4×10^{-3}

↑
Nu-1825
 10^{-3}

↑
Nu-1825
 2×10^{-3}

C

FIG. 3. ACTION ON GUINEA PIG'S TRACHEAL RINGS
From left to right at: A. Epinephrine, gm/cc, 5×10^{-3} ; 10^{-3} ; 2×10^{-3} . B. Nu-1408, gm/cc, 10^{-3} ; 2×10^{-3} ; 4×10^{-3} . C. Nu-1825, gm/cc, 10^{-3} ; 2×10^{-3} . All records from the same preparation. Time in minutes.

experimental details have been described previously (13). The data presented graphically in figure 4 represent the averages of at least three experiments with

TABLE 2
Action on isolated organs

COMPOUND	RELATIVE ACTIVITY ON TRACHEAL RINGS	RELATIVE ACTIVITY ON INTESTINAL MOTILITY	RELATIVE ACTIVITY ON ISOLATED HEART
Epinephrine.....	1	1	1
Nu-1408.....	1/20	1/20	1/25
Nu-1825.....	1/200	1/80	1/100
'Neosynephrine'.....		1/50	
Nu-1683.....	1/400	1/1000	1/1000
Nu-2013.....	1/300	1/500	1/1000
Nu-2014.....	1/800	1/4000	1/4000
Nu-1896.....	1/4000	1/4000	1/800
Ephedrine.....	1/1000	1/2000	
Nu-1272.....		Spasm	
Nu-1410.....		<1/10,000	
'Propadrine'.....		1/5,000	
Nu-1318.....		<1/10,000	

Relative Rate of Oxidation, Percent of Tyramine

10 20 30 40 50 60 70 80 90 100 110 120

Tyramine

Epinine

Nu-1408

Epinephrine

Neosynephrine

Nu-1683

Phenethylamine

Nu-1472

Nu-1473

Nu-1318

Nu-1272

FIG. 4. RATE OF OXIDATION AS PERCENT OF TYRAMINE OF MONO- AND DI-AMINES BY AMINE OXIDASE IN GUINEA PIG LIVER BREI

each compound. The diamines having ethyl side chains are readily oxidized whereas those with a propyl side chain resist oxidation. A certain quantitative correlation between mono- and diamines is apparent. Nu-1408 is oxidized at

about the same rate as its hydroxy analog, epinephrine, but more slowly than 'Epinine' (13). Similarly, the oxidation of Nu-1683 and Nu-1472 proceeds at the same speed as that of their corresponding mono-amines, 'Neosynephrine' and phenylethylamine. It seems that the second amino group is not attacked by amine-oxidase. This observation is in accordance with Beyer's findings (14), that amine-oxidase is ineffective against an amino group attached to a secondary carbon-atom. The effect of diamine-oxidase on these diamines remains to be investigated.

SUMMARY

A series of 25 aromatic and aliphatic alkylene- α,β -diamines have been studied.

Most of the arylalkylenediamines may be considered as derived either from alkanolamines by substituting an amino group for the alcoholic hydroxyl group or from alkylmono-amines by introducing a second amino group.

The diamines are usually less toxic but also less potent pressor substances than the corresponding alkanolamines but are in many instances as potent as the corresponding alkylmono-amines.

Of the 5 major variations in position of the amino groups in the alkyl chain—phenylethylenediamine, phenyl-1,2-propanediamine, phenyl-2,3-propanediamine, phenyl-2,3-butanediamine and 2,3-alkyldiamine only compounds of the first type such as 1-(3,4-dihydroxy phenyl)-N²-methylethylenediamine (Nu-1408), 1-(*m*-hydroxyphenyl)-N²-methylethylenediamine (Nu-1683), its di-isomer (Nu-2013) and the corresponding primary diamines, Nu-1825 and Nu-1896, possess sufficiently high pressor activity to be of interest. Nu-1683 and Nu-2013 resemble ephedrine in pressor potency and duration of action, but are one-third as toxic, lack the central stimulating action and do not produce tachyphylaxis.

The phenylethylenediamines are oxidized by amine oxidase at the same rate as the corresponding mono-amines.

ACKNOWLEDGEMENT. The technical assistance of Mr. H. F. Brumbach, Miss E. Hagan and Miss D. Oliver is gratefully acknowledged.

We are indebted to Dr. W. Lauter of the Pharmaceutical Development Laboratory for carrying out the stability test with Nu-1408.

REFERENCES

1. HARTUNG, W. H.: *Ind. and Eng. Chem.*, **37**: 126, 1945.
2. FEIST, F., AND ARNSTEIN, H.: *Ber. d. dtsh. Chem. Ges.*, **28**: 425, 1895.
3. JAEGER, F. M., AND VAN DIJK, J. A.: *Proc. Acad. Sc. Amsterdam* **44**: 26, 1941.
4. DAKIN, H. D., AND WEST, R.: *J. Biol. Chem.* **78**: 757, 1928.
5. BENOIT, G., AND HERZOG, A.: *Bull. Sci. Pharmacol.* **42**: 34, 1935.
6. FUNKE, A., AND BOVET, D.: *Compt. rend. Soc. Biol.* **141**: 327, 1947.
7. DUSCHINSKY, R., DOLAN, L. A., RANDALL, L. O., AND LEHMANN, G., *J. Am. Chem. Soc.* **69**: 3150, 1947.
8. DUSCHINSKY, R., AND DOLAN, L. A.: *J. Am. Chem. Soc.*, **67**: 2079, 1945.
9. DUSCHINSKY, R., AND DOLAN, L. A.: *J. Am. Chem. Soc.*, **68**: 2350, 1946.
10. GUNN, J. A., AND GURD, M. R.: *J. Physiol.* **97**: 453, 1940.
11. HJORT, A. M., DEBEER, E. J., AND RANDALL, L. O.: *THIS JOURNAL* **71**: 105, 1941.
12. CASTILLO, J. C., AND DEBEER, E. J.: *THIS JOURNAL*, **90**: 104, 1947.
13. RANDALL, L. O.: *THIS JOURNAL*, **88**: 216, 1946.
14. BEYER, K. H., AND LEE, W. V.: *THIS JOURNAL*, **74**: 155, 1942.



George Barclay Wallace

George Barclay Wallace

1874-1948

The members of the Society for Pharmacology and Experimental Therapeutics have experienced a sadness and suffered a loss in the death of Dr. George Barclay Wallace which occurred in Bellevue Hospital as the result of a cerebral hemorrhage, January 15, 1948.

Dr. Wallace was one of the few remaining men of the older order who had not only watched pharmacology in this country emerge from empirical therapeutics and establish itself as a pure and as an applied science, but he had much to do with this transition through research, teaching and the furtherance of various organizations and publications of a pharmacological nature.

The research interests of Dr. Wallace embraced such widely separated intellectual adventures as the pharmacology of the volatile oils, a study of body water, the distribution of various electrolytes in the animal organism, the mode of action and comparative efficacy of various saline cathartics, the influence of the cyanides on cellular respiration and the glycosuria which develops in the higher animals during intoxications by uranium nitrate. In the latter investigation he demonstrated that such a glycosuria was not associated with a hyperglycemia but made its appearance as the renal injury developed from the use of this heavy metal. The action of such metallic poisons stimulated his interest. His last research contribution concerned itself with the absorption, distribution in the tissues and the elimination of iodine derivatives. This brief account is sufficient to indicate the breadth of Dr. Wallace's research interests and also serves to emphasize the general understanding he had of this science and its relationship to medicine as a whole. Dr. Wallace was an accurate and a cautious workman. His deductions were never expansive beyond the realm of supporting data and his studiousness not only made him familiar with the accumulated literature of a subject but assured earlier students full recognition for their part in the solution of those problems which he undertook.

As teacher, Dr. Wallace stimulated and gave freedom to that blessed attribute possessed by some students, designated thought. This came about not by the use of processes commanding memory of dead facts but through experimentation and suggestion on his part as the experiment progressed. This gave life to learning and substituted wonder and inquisitiveness for the static knowledge of textbook material. In his teaching of pharmacology Dr. Wallace kept ever in mind that he was imparting information concerning the action of various chemical substances to medical students in order that these students as physicians might take such learning and with some degree of scientific assurance apply it to tissues in the distress and imbalance of an illness in the hope of effecting tissue readjustments back to or towards the normal. He was concerned with the use of pharmacology as a scientific tool in the making of good doctors. In his teaching he therefore gave emphasis to applied pharmacology. He gave himself freely to his students. They in turn gave to him a fine respect and a deep affection

Dr. Wallace was one of the initial group of teachers and investigators to form the Society for Pharmacology and Experimental Therapeutics. As this organization grew and became more complex he served with great faithfulness on its various committees and became its president in 1929. He had a continuous and sustained interest in the journal of this society and after periods during which he acted as associate editor, he became for a number of years Editor-in-Chief. Interest of a similar order was shown by Dr. Wallace for the Society for Experimental Biology and Medicine, for the Proceedings of this society in the form of its journal, for the Harvey Society and the outstanding lectureships which it sponsored and for the New York Academy of Medicine. For the Harvey Society and the Academy Dr. Wallace served as guide and councilor not only in a specifically designated sense but through the broader relationship of such organizations to the medical and scientific life of New York City. His judgment in such matters was both sound and conservative and gave him in a measure the position of elder statesman for medical affairs in this metropolis. This influence expressed itself freely and effectively for the development of his institution, the Medical College of New York University. In the anticipated expansion of this school as the Bellevue Medical Center this influence exerted itself in an effective fashion.

Over and above specific attainment George Wallace was possessed of a Something that was good and fine and noble. His friendship was of a democratic order, genuine and lasting. He knew no sham or pretense. He stood squarely for the right as his reason gave him the ability to see it. He was a man of strength and poise and unassumed dignity.

WM. DEB. MACNIDER

THE DETERMINATION OF 3-(ORTHOTOLOXYL)-1,2-PROPANEDIOL (MYANESIN) IN BODY FLUIDS AND TISSUES, AND ITS DISAPPEARANCE FROM THE BLOOD FOLLOWING INTRAVENOUS INJECTIONS IN THE DOG

ELWOOD TITUS, STANLEY ULICK AND ARTHUR P. RICHARDSON¹

Division of Pharmacology, Squibb Institute for Medical Research, New Brunswick, New Jersey

Received for publication September 8, 1947

It has been recognized for some time that alpha ethers of glycerine produce paralysis of a variety of species of animals, presumably by depression of the central nervous system (1, 2). Interest in this group of compounds has been revived by the investigations of Berger and Bradley (3, 4), who found 3-(orthotoxyl)-1,2-propanediol (Myanesin) to be the most potent and the safest of the compounds they have examined. Mallinson (5) has used Myanesin in a series of 112 cases as an adjunct to anesthesia, and has suggested that it may serve as a satisfactory substitute for curare.

In order to provide the analytical methods required for further pharmacological investigation of myanesin and its analogues, two procedures have been developed. The more useful of these depends on the fact that under proper conditions, phenolic ethers can be made to couple with the more reactive diazonium compounds. A somewhat less sensitive method, of interest because it utilizes another portion of the myanesin molecule, involves periodate oxidation of the glycerine side chain to formaldehyde (6), which may then be determined colorimetrically with chromotropic acid (7).

PROCEDURE FOR COUPLING METHOD

Reagents

Chloroform C. P. redistilled

2N Sodium Hydroxide

85 per cent Phosphoric Acid C. P., washed before use with an equal volume redistilled chloroform.

Diazotized 2,4-dinitroaniline (8). One gram of 2,4-dinitroaniline (Eastman Kodak, purified by recrystallization from ethanol-water) is dissolved in 5 ml. of concentrated sulfuric acid by warming gently on the steam bath. The solution is then cooled to 0°C in an ice-salt bath. Half a gram of sodium nitrite is dissolved in another 5 ml. portion of concentrated sulfuric acid which has been cooled to 0°C, and the two solutions are mixed. The sulfuric acid solution of dinitroaniline and sodium nitrite is kept at 0°C and stirred mechanically for an hour, during which 20 ml. of 85 per cent phosphoric acid is added dropwise. Six hundred seventy milligrams of urea are then added to decompose excess nitrite, and the solution of diazotized 2,4-dinitroaniline is diluted to 100 ml. with 85 per cent phosphoric acid. The reagent is prepared by diluting 8 ml. of this stock solution to 100 ml., corresponding to 800 micrograms of dinitroaniline per ml. Both solutions should be stored in the refrigerator. They are stable for at least two months.

¹ Present Address Emory University, School of Medicine, Emory University, Georgia.

Standard solution of Myanesin. 10 micrograms per ml. in 85 per cent phosphoric acid. Store in refrigerator.

Procedure

A sample of 0.5 ml. of plasma or urine is placed in a glass stoppered centrifuge tube containing 9 ml. chloroform and 5 ml. 2N NaOH, (which prevents the extraction of interfering substances), shaken mechanically for ten minutes, and centrifuged. The gel and aqueous phase are removed by suction through a piece of glass tubing drawn out to a capillary. A 7 ml. aliquot of the chloroform extract is pipetted into another glass stoppered centrifuge

TABLE I
Recovery of Myanesin from plasma

MYANESIN ADDED TO PLASMA	CTA METHOD		COUPLING METHOD	
γ /ml.	γ /ml.	%	γ /ml.	%
10	11.5	115	9.3	93
20	19	97	19	95
40	35	88	37	93
60	54	89	66	109
80	79	99	87	109
100	93	93	112	112

TABLE II
Recovery of Myanesin from urine

MYANESIN ADDED TO URINE	CTA METHOD		COUPLING METHOD	
γ /ml.	γ /ml.	%	γ /ml.	%
10	9.0	90		
20	19	94	17	85
40	36	90	37	94
60	55	92	59	99
80	61	77	75	94
100	90	90	101	101

tube containing 7 ml. of 85 per cent phosphoric acid, shaken mechanically for ten minutes and centrifuged. After removal of the upper layer, a 6 ml. aliquot of the remaining phosphoric acid layer is pipetted into an Evelyn colorimeter tube and 0.5 ml. of diazotized 2,4-dinitroaniline reagent containing 800 micrograms per ml. of 85 per cent phosphoric acid is added. The colorimeter tube is placed in a boiling water bath for 20 minutes, then cooled in an ice-bath. Transmission of the red-colored solution is read in an Evelyn colorimeter using a 520 filter and the 6 ml. aperture. Eighty-five per cent phosphoric acid is used for blank setting of the instrument. Samples have a stable color for at least 24 hours when stored in the refrigerator.

Calculation of Results

A standard curve is obtained by heating 6 ml. portions of phosphoric acid containing from 5 to 50 micrograms of Myanesin with diazonium reagent as described above. With the Evelyn colorimeter, such curves show a linear relationship between extinction and

quantity of Myanesin, up to approximately 20 micrograms of the drug. With the Beckman spectrophotometer, the linearity is retained up to about 50 micrograms, the optical density (extinction per cm. depth of solution) per microgram of myanesin being 0.017. The smallest amount of Myanesin which can be determined with significance is 2 micrograms per ml. of phosphoric acid.

To obtain micrograms of Myanesin per ml. of original plasma or urine when the above pro-

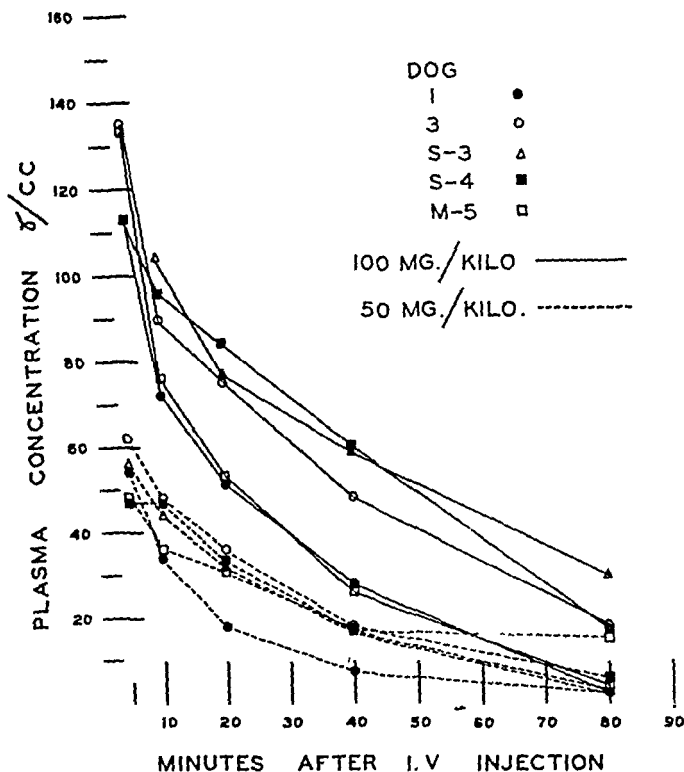


FIG. 1. Rate of disappearance of Myanesin from the plasma of dogs after intravenous injection. All analyses made with coupling method.

cedure is used, the amount of Myanesin corresponding to the net extinction after subtraction of blank values is multiplied by

$$2 \times \frac{9}{7} \times \frac{7}{6} \times \frac{1}{.80} = 2.91$$

to correct for the aliquots. Since the distribution constants of 3.8 for Myanesin in $\text{CHCl}_3/0.1\text{N NaOH}$ and 4.5 for the same substance in 85 per cent $\text{H}_3\text{PO}_4/\text{CHCl}_3$ permit the recovery of 80 per cent of the original quantity of Myanesin over the double extraction step, the factor of $1/.80$ is required in the above expressions.

PROCEDURE FOR CHROMOTROPIC ACID (CTA) METHOD

Reagents

Chloroform C. P., redistilled

1.0N Sodium Hydroxide

0.35N Sodium Bicarbonate

0.25 per cent Potassium Periodate. 250 mgms. of C. P. KIO_4 made up to 100 ml. in 0.1N H_2SO_4 . This solution is stable indefinitely.

5.0N Hydrochloric Acid.

0.1N Sodium Arsenite. Reagent grade.

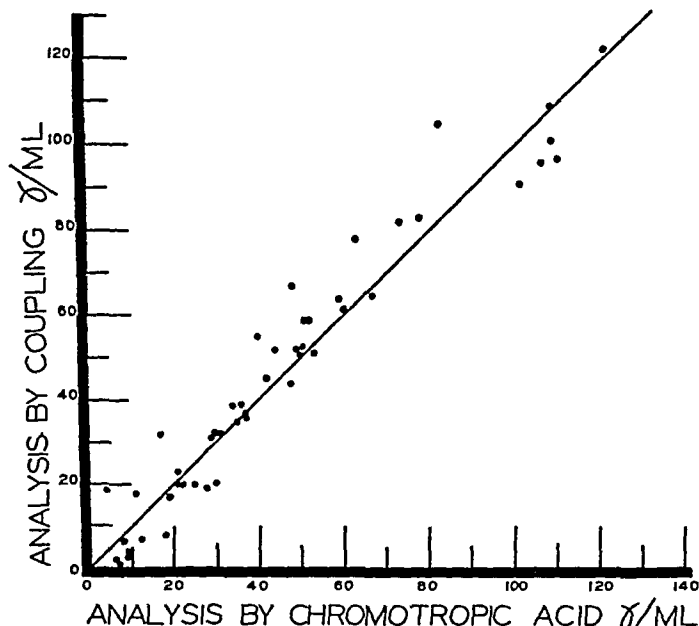


FIG. 2. Comparison of analyses of plasma concentration of myanesin in dogs as done by coupling method and chromotropic acid method. Each point represents a single analysis by each method.

12.5 Molar Sulfuric Acid.

Chromotropic Acid. 1.0 grams of 1,8-dihydroxy naphthalene-2,6-disulfonic acid (Eastman Kodak) dissolved in 20 ml. of water. Shortly before use one part of this stock solution is diluted with nine parts of 12.5 molar H_2SO_4 .

Procedure

One ml. of urine or plasma plus 0.1 ml. of 1.0N NaOH is shaken vigorously for three minutes with 25 ml. of chloroform in a 50 ml. glass stoppered bottle. After settling the upper layer is drawn off by means of a capillary pipette attached to an aspirator, and a 20 ml. aliquot of the lower layer pipetted into a 50 ml. Erlenmeyer flask. The chloroform is evaporated from a number of samples simultaneously by placing the flasks in a vacuum desiccator. To each residue is added 0.4 ml. of 0.35N NaHCO_3 and 0.4 ml. KIO_4 solution.

After standing one hour, 0.1 ml. 5N HCl and 0.2 ml. of 0.1N NaAsO₂ are added to each flask and the solution allowed to stand for twenty minutes to ensure complete fading of the temporary iodine color. Four ml. of chromotropic acid reagent are added to each flask and the contents transferred to test tubes, which are then immersed in a boiling water bath for one hour. Since varying amounts of illumination can cause irregularities in the development of color, the bath is best protected from direct light.

When the reaction has been completed, the tubes are cooled to room temperature, and the extinction measured in a Beckman spectrophotometer at 570 m μ using cuvettes of one cm. square cross-section.

Calculation of Results

A standard curve obtained with a series of samples containing from 5 to 100 micrograms of Myanesin in 0.4 ml. of 0.35 molar NaHCO₃ is linear, the optical density per microgram of Myanesin being 0.0099. This value has generally been found to be reproducible within

TABLE III

Apparent volume of distribution and urinary excretion of Myanesin following intravenous injection in dogs

DOG NO.	50 MGMS PER KILO		100 MGMS PER KILO	
	Apparent Volume of Distribution Per Cent of Body Weight	Per Cent Recovered From Urine	Apparent Volume of Distribution Per Cent of Body Weight	Per Cent Recovered from Urine
1	65.8	1.72	65.7	0.72
3	68.5	1.26	62.9	0.34
S-3	78.1	0.16	68.0	0.09
S-4	76.8	0.11	70.5	0.09
M-5	80.5	0.31	60.6	0.15
Mean... ..	73.9	0.71	65.5	0.27

5 per cent. When allowance is made for the 4/5 aliquot of chloroform, the concentration of Myanesin in the original sample becomes $126 \times D$, where D represents the optical density of the solution after subtraction of the blank.

The smallest concentration which can be practically measured is 5-6 micrograms per ml. corresponding to values of 0.04-0.05 for D.

Although the coupling procedure is applicable to both blood and urine, the variable blank values encountered make the CTA method unsatisfactory for determinations in urine.

Tables I and II record the recoveries of Myanesin added to plasma and urine.

In order to study the rate of disappearance of Myanesin from the blood, doses of 100 and 50 mgm. per kilogram were administered intravenously to a series of normal dogs. Plasma levels determined at various time intervals after administration are plotted in Figure 1. The rapid disappearance of the compound from the blood stream is not unexpected in view of the short lived activity of Myanesin noted by Berger and Bradley (3), and our observations that the anesthetic effect of doses of 50 mgm. per kilogram persisted for only 10 minutes and that of the larger doses for 20 to 30 minutes.

In Figure 2 the result of each plasma level determination by the coupling

method is plotted against the value obtained with chromotropic acid for the same sample. In general, the results of the two methods agree within about 10 per cent. Since the two determinations involve different functional groups in different parts of the molecule, such agreement would indicate that Myanesin and not a degradation product is being measured.

The total amounts of Myanesin excreted in urine over a period of 80 minutes after administration of the drug are recorded in Table III. In no case was more than 2 per cent of the original dose recovered in urine. Apparent volume of distribution calculated from the data of Figure 1 and recorded in the same table suggest that the drug is distributed throughout the body water.

SUMMARY

Analytical methods for 3-(orthotoloxyl)-1,2-propanediol (Myanesin) involving coupling with diazotized 2,4-dinitroaniline and colorimetric determinations of the formaldehyde resulting from periodate oxidation have been developed. Of these, the former is more generally applicable to plasma and urine. The latter, which may be used for plasma determinations, gives results in agreement with the coupling procedure.

The rate of disappearance of Myanesin from the blood of dogs receiving doses of 50 and 100 mgm. per kilogram has been measured.

REFERENCES

1. FILIPPI, E. AND RODOLICO, L.: *Arch. farmacol. sper.*, **11**: 1, 1909
2. GILBERT, A. AND DESCOMPS, P.: *Compt. rend. soc. biol.*, **69**: 145, 1910
3. BERGER, F. M. AND BRADLEY, W.: *Brit. J. Pharmacol.* **1**: 265, 1946
4. BERGER, F. M. AND BRADLEY, W.: *Lancet*, **252**: 97, 1947
5. MALLINSON, F. B.: *Lancet*, **252**: 98, 1947
6. REEVES, R. E.: *J. Amer. Chem. Soc.*, **63**: 1476, 1941
7. MACFADYEN, D.: *J. Biol. Chem.*, **158**: 107, 1945
8. SCHOUTISSEN, H. A. J.: *J. Amer. Chem. Soc.*, **55**: 4531, 1933

A PYREX APPARATUS FOR THE PERFUSION OF THE CORONARY CIRCULATION OF MAMMALIAN HEARTS*

FREDERICK F. ANDERSON AND BRADFORD N. CRAVER

*Research Department, Division of Pharmacology, Ciba Pharmaceutical Products, Inc.,
Summit, New Jersey*

Received for publication January 28, 1948

The classic method of Langendorff (1) for the perfusion of hearts from warm-blooded animals has been widely employed and variously modified since. Uhlmann and Nobile (2) in presenting an excellent arrangement for exchanging perfusion fluids without altering the pressure, have reviewed the earlier work. More recently the careful studies of Chenoweth and Koelle (3) have re-emphasized and extended earlier observations in their delineation of the requisites for a long-lived preparation. These requisites have been carefully incorporated in the present apparatus which does, we believe, represent an efficient and compact unit for their realization (Fig. 1).

The apparatus, of Pyrex glass and Tygon tubing, employs washed air: (a) for agitating the fluid in the constant temperature bath; (b) for circulating that fluid through the double-walled chamber enclosing the heart; (c) for recycling the perfusion fluid. The apparatus provides for two separate fluid circulations: (1) that of the perfusion fluid; (2) that of the warming fluid, which is distilled water to obviate gumming of the apparatus. The upper chamber supported by two wide brass bands clamped to the stand contains distilled water maintained at 39°C., through which passes the perfusion fluid in a glass coil. Inserted into the top of this chamber are three things, visible in the picture but omitted in the diagram: (1) a bent glass tube carrying air to the bottom of the chamber to act as an agitator; (2) a 250 Watt immersion heater; (3) a sensitive thermostat to control the heater. Several types of thermostat or heater would obviously be satisfactory provided they could be fitted into place between the coils. The double-walled chamber surrounding the heart is attached to the upper chamber by a 45/50 standard taper glass joint held in position by two wire springs which are clearly visible in the picture. A 55/50 would allow more room for sliding the lower chamber into position over the heart. The perfusion fluid is put into the aspirator bottle on top of the stand. Into its top pass: (1) the perfusion fluid return (L) described below; (2) an ordinary "cold finger" to lessen evaporation (see sketch); and, (3) a tube through which bubbles a constant fine stream of 95% oxygen and 5% carbon dioxide. This maintains oxygenation and the pH of the perfusion fluid (for formula see (1)). The course of the fluid as it emerges from the bottle at A is indicated by the consecutive letters of the alphabet. For the sake of simplicity the Tygon tubing connecting the various pieces of apparatus has been omitted in the diagram. The fluid enters at B, a filter

* Presented as a static demonstration at the Thirty-First Annual Meeting of the Federation of American Societies for Experimental Biology.

ordinarily stoppered at the top and containing glass wool, whence it emerges at C. Thence it passes behind the board (seen in the photograph but omitted from the diagram; it is attached below the stand upon which the perfusion bottle rests) to emerge again after having been exposed to an ultra-violet lamp (General Electric, 8 Watt germicidal lamp, 11½ inches long). The glass tube carrying the liquid in front of the lamp is permeable to ultra-violet.¹ The fluid enters the

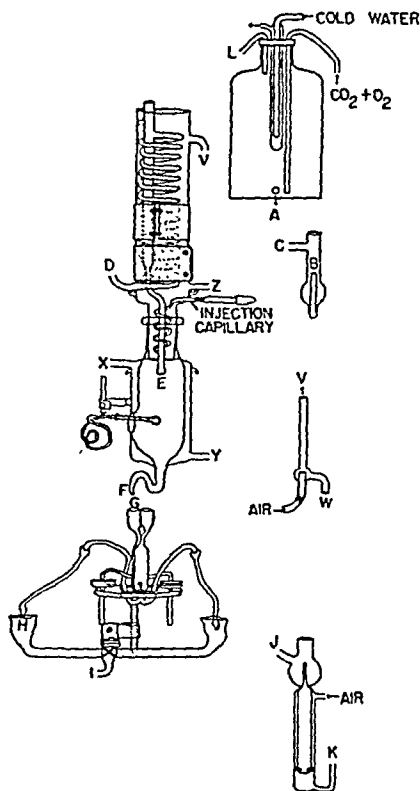


FIG. 1. DIAGRAM AND PHOTOGRAPH OF CORONARY PERFUSION APPARATUS

warming chamber at D and spirals upward to terminate in a well, the upper end of which projects in the diagram above the top of the warming chamber. In preparation for an experiment that well must be filled with fluid, by keeping the finger over the exit of the coil at E, and then stoppered. The well allows bubbles of gas, released as the gassed fluid is heated in the warming bath, to rise to its top rather than to be carried down through the exit at E, as would

¹ This special tubing, 12 mm. in internal diameter, was supplied through the courtesy of General Electric Company, Schenectady, New York.

occur if the small bore of the coil were maintained throughout. The outer end of the "injection capillary" is a medicine dropper connected to the main chamber

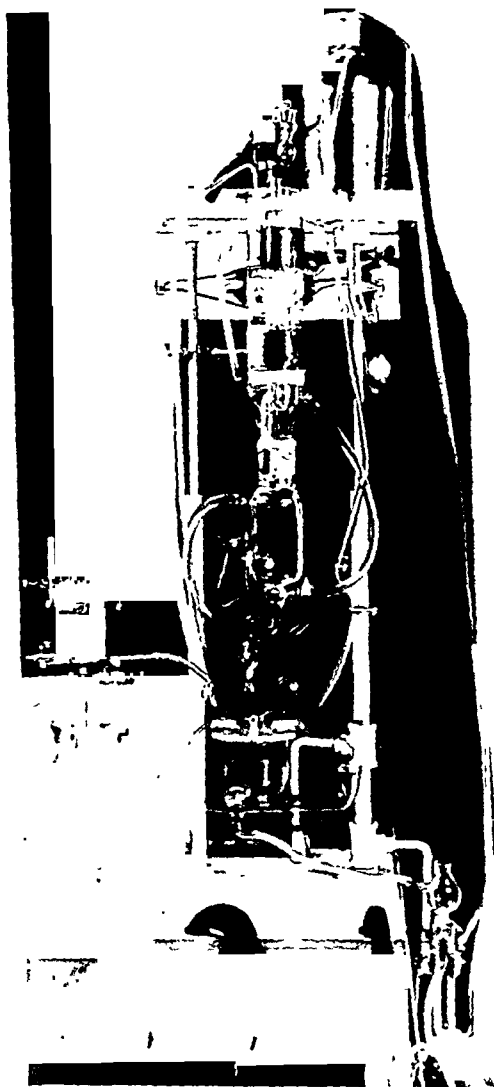


FIG. 1. (Continued)

by a piece of rubber tubing through which drugs can be injected. Slow squeezing of the bulb washes the drug completely into the exit of the coil just above

the heart. Sudden squeezes, by their considerable, if momentary, effect on the perfusion pressure would alter the recording of the contractions.

The heart is removed in the usual manner and an aortic cannula tied in place. This is attached by thick-walled rubber tubing to E. Two skilled operators can

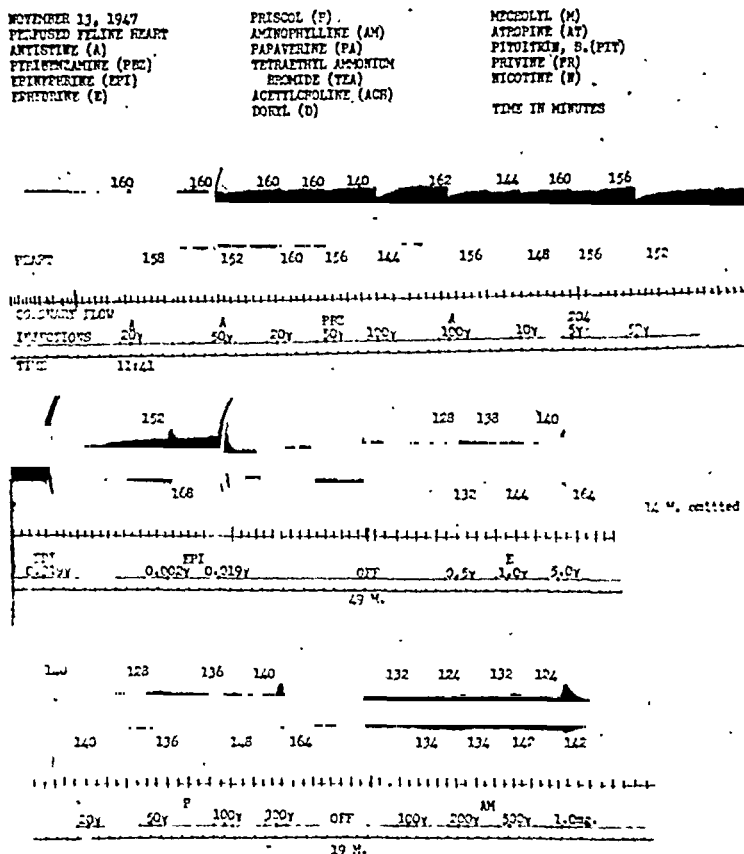


FIG. 2. This tracing represents a study of the effects of various drugs commonly employed as pharmacological "tools" in this type of preparation. To save space portions of the record have been omitted. Numbers above and below "HEART" equal cardiac rates per minute before and after injections. Coronary flow is in 13 ml. increments.

easily hold the time interval during which the coronary circulation is absent below one minute, and indeed occasionally below half a minute. If the time exceeds one minute an irregular and short-lived preparation is almost sure to result. The double-walled chamber for surrounding the heart is then fitted

into place and fastened with two springs attached to the four glass hooks shown in the diagram. The glass pulley, attached to a flexible lead rod, is passed in through a port in the chamber. The S-shaped tube F at the bottom prevents splashing, avoids convection currents within the chamber and allows an even outflow of the perfusion fluid. The stromuhr below F is essentially a carefully balanced tipping device, supported at but two points. Only the support toward the reader is visible and it is shown in the diagram lifted slightly out of its bearing to make clearer that it is a simple, glass hook. It operates as follows: The fluid from F drips into the chamber G. When the chamber has been filled it drains

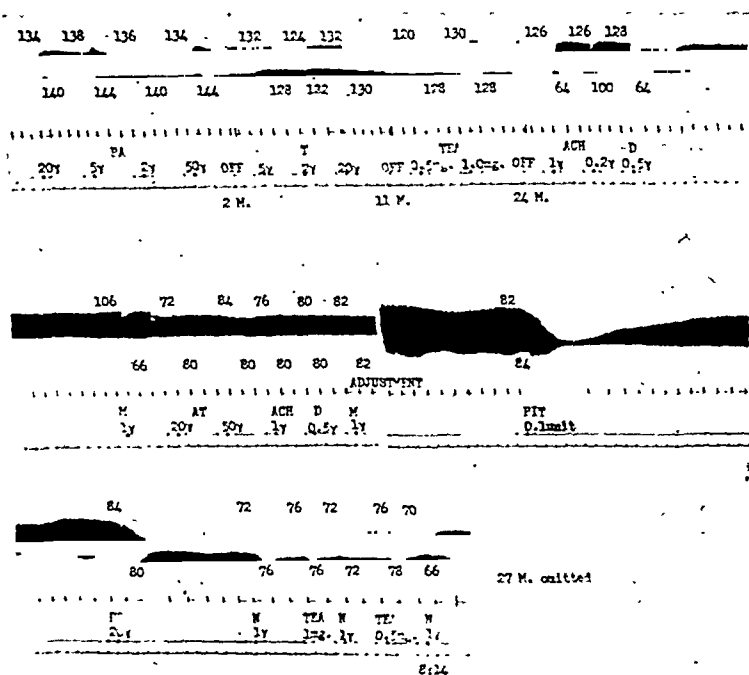


FIG. 2. (Continued)

by siphonage at H. That shifts the stromuhr's center of gravity so it tilts to the left thereby bringing the other chamber under F. This fills in turn and siphons out at the right. As the chamber tilts, glass strikers hit rubber membranes connected to a Marey tambour arranged to record on the kymographic record. From the bottom of the collecting U-tube I the perfusate flows into an air pump at J. Air enters at the side in a slow, constant stream and escapes upward when the pump is empty. The fluid flows in at J and down through the neck into the chamber containing a float with an upper tip which serves to guide its rise. As fluid collects in the pump the float rises (in the diagram it is repre-

NOVEMBER 24, 1947
 PERFUSED HEART OF GUINEA PIG
 ASSAY OF PRISCOL SOLUTIONS -
 WARBURG STUDY OF DETOXICATION
 INJECTIONS REPRESENT 50% OF
 PRISCOL ASSUMING NO DESTRUCTION

NUMBERS ABOVE & BELOW "HEART"
 CARDIAC RATES/MINUTE BEFORE AND
 AFTER INJECTIONS
 CORONARY FLOW IN 13 ml. INCRE-
 MENTS
 TIME IN MINUTES

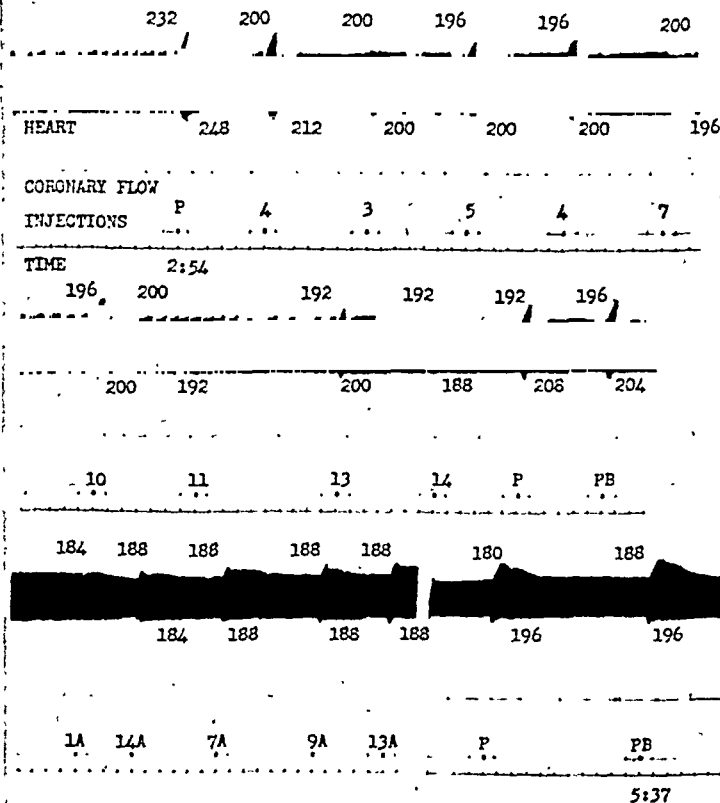


FIG. 3. This tracing represents an assay of Priscol solutions secured from a Warburg study of possible detoxication by rat hepatic and renal slices. The original concentration of 10 mg./ml. of Priscol was so diluted that each injection of 0.05 ml. would have contained 50 % of Priscol, had none been destroyed. Control solutions were prepared in the same manner. The solutions studied were as follows:

Controls

Priscol in 0.9% saline (P)

Priscol in buffer, not incubated (PB)

Warburg Samples

- (No. 3: Buffer with kidney (3)
- " 4: Priscol with kidney (4)
- " 5: Priscol with liver (5)
- " 7: Priscol with liver, then kidney (7)
- " 10: Priscol in buffer, incubated (10)
- " 11: Buffer alone, incubated (11)
- " 13: No. 5 (13)
- " 14: No. 7 (14)
- " 1A: Buffer with liver (1A)
- " 7A: Priscol incubated 2 hours with liver (7A)
- " 9A: Priscol incubated 3 hours with liver (9A)
- " 13A: Priscol incubated 4½ hours with liver (13A)
- " 14A: Priscol incubated 6 hours with liver (14A)

sented in the raised position) and two ground glass surfaces are brought into juxtaposition. The air, with its ordinary escape thus cut off, forces the fluid in the pump out K and up a tube into the perfusion bottle at L.

The second circulation, that of the warming fluid, may now be briefly described. It begins at Z and follows letters backward in the alphabet. The water emerges from the upper chamber at Z and flows into the lower double-walled chamber at Y. It flows out at X and passes into an air lift at W. Air carries the warming fluid out the top and back into the upper chamber at V, to complete this circulation.

With this apparatus the hearts of six species have been successfully perfused: those of the cat, rabbit, guinea pig, hamster, rat and dog. With the last, however, even the hearts of puppies proved impracticably large for the apparatus to yield satisfactory experiments. Experiments were deemed satisfactory if regular contractions were maintained throughout and the amplitude of the contractions at the end of the experiment were at least 50% that at the beginning. By this criterion the most sensitive hearts, those of the rat and hamster, functioned well for at least three to four hours. Drugs were, of course, injected during that interval. To minimize a progressive contamination with drugs of the recycled perfusion fluid, at least 13 ml. of the fluid was collected and discarded after each injection of a drug. This volume is that contained in one chamber of the flowmeter. The hearts of cats and rabbits usually functioned well for 8 hours or longer, and some feline hearts were followed as long as 16 hours. After such an interval the edema of the heart was considerable but an investigation of the potential value to such preparations of blood and various substitutes therefor, made it clear that their desirable osmotic effects were more than negated by the increases in perfusion pressure necessitated by their viscosity. In all our experiments the perfusion pressure has been kept within the range of 37-50 centimeters of water. The preparations herein described are not only valuable for screening new drugs, but, because of their duration, permit studies of cardiac metabolism. Figures 2 and 3 represent typical experiments secured with this apparatus.

SUMMARY

An all Pyrex apparatus for coronary perfusion by the technique of Langendorff has been described. Its application to the hearts of six species: rat, rabbit, guinea pig, cat, hamster and dog, has been discussed.

REFERENCES

- (1) LANGENDORFF, O.: *Arch. ges. Physiol.* 61: 291, 1895.
- (2) UHLMANN, F., AND NOBILE, F.: *Arch. f. exp. Path. u. Pharm.* 192: 189, 1939.
- (3) CHENOWETH, M. B., AND KOELLE, E. S.: *J. Lab. Clin. Med.* 31: 600, 1946.

A METHOD FOR THE QUANTITATIVE ESTIMATION OF THEOPHYLLINE IN BLOOD AND URINE:

APPLICATION TO THE DOG¹

ALBERT J. PLUMMER

Department of Pharmacology, Boston University School of Medicine

Received for publication January 31, 1948

There is need for a specific and sensitive method for the quantitative determination of theophylline in blood and urine in order to follow the course of the substance in the body and to control more properly the method of administration of this commonly used drug to secure its optimal effects.

A method applicable to blood has been described by Truitt et al (1) which is based upon the spectrophotometric measurement of the color produced by the coupling of theophylline with Fast Blue 2 B after preliminary treatment with concentrated alkali.

The method to be described here is based upon a reaction reported by Plummer and Mendenhall (2). Its advantages are high sensitivity, technical simplicity, stability of reagents and applicability to both blood and urine. In addition it is not interfered with by caffeine, theobromine, uric acid, ethylene diamine or sodium acetate. Since the last two substances are commonly added to theophylline when it is administered therapeutically it is an advantage that their removal is not a necessary preliminary to the quantitation of aminophylline. The method also is not interfered with by any normal blood property or constituent.

METHOD

1. Add 5 cc. of a saturated solution of copper acetate in methyl alcohol to 4 cc. of a methyl alcohol solution of theophylline in a 15 cc. centrifuge tube.

2. Let stand tightly stoppered for 4 hours to insure complete precipitation of the theophylline copper compound.

3. Centrifuge for 15 minutes at 1000 R.P.M.

4. Decant the supernatant liquid, drain and wash the precipitate with 5 cc. of methyl alcohol.

5. Centrifuge as before and again decant and drain.

6. Dissolve the precipitate in 4 cc. 0.2 N sulfuric acid.

7. Add 0.5 cc. of potassium iodide solution (1 Gram to 1 cc. water).

8. Titrate the liberated iodine with 0.02 N sodium thiosulfate using soluble starch solution as an indicator. 1 mgm. of theophylline is equivalent to 0.57 cc. of 0.02 N sodium thiosulfate.

The method was applied to various concentrations of theophylline in methyl alcohol. The results are tabulated in Table 1.

¹ Aided by a grant from the American Medical Association Council on Pharmacy and Chemistry.

Application of Method to Blood.

1. Deproteinize by adding 25 parts of blood to 40 parts of 13% trichloroacetic acid.
2. Allow to stand 20 minutes; filter or centrifuge.

TABLE 1

Volume of 0.02 N sodium thiosulfate which was required to titrate 4 cc. of methyl alcoholic solution of theophylline of various concentrations. Each value is the average of five determinations

MGM THEOPHYLLINE/100 CC.	CC 0.02 N SODIUM THIOSULFATE
6	.14
10	.24
20	.47
40	.94
80	1.86
100	2.28
120	2.71

TABLE 2

Determination of theophylline added to blood

MGM. THEOPHYLLINE ADDED PER 100 CC BLOOD	MGM. THEOPHYLLINE FOUND PER 100 CC. BLOOD
.20	.18
.20	.21
.50	.50
.50	.48
.80	.77
.80	.79

TABLE 3

Determination of theophylline added to urine

MGM THEOPHYLLINE ADDED PER 100 CC URINE	MGM. THEOPHYLLINE FOUND PER 100 CC. URINE
.25	.28
.25	.25
.50	.48
.50	.47
1.00	.97
1.00	.98

3. Render just alkaline to litmus with 2.5 N sodium hydroxide.
4. Add 10 cc. of a phosphate buffer of pH 8.0. The final pH must lie between 7.3 and 8.2.
5. Extract the theophylline by shaking the buffered filtrate with three 20 cc.

portions of a mixture of 3 volumes of chloroform and 1 volume of isopropyl alcohol² for 5 minutes each extraction.

6. Evaporate the combined extracts just to dryness on a water bath.

7. Dissolve the residue in methyl alcohol warming on the water bath to facilitate solution.

8. Transfer the solution to a 15 cc. graduated centrifuge tube keeping the final volume of methyl alcohol between 0.3 and 0.5 cc. If this is exceeded warm until it is reduced to this range by evaporation.

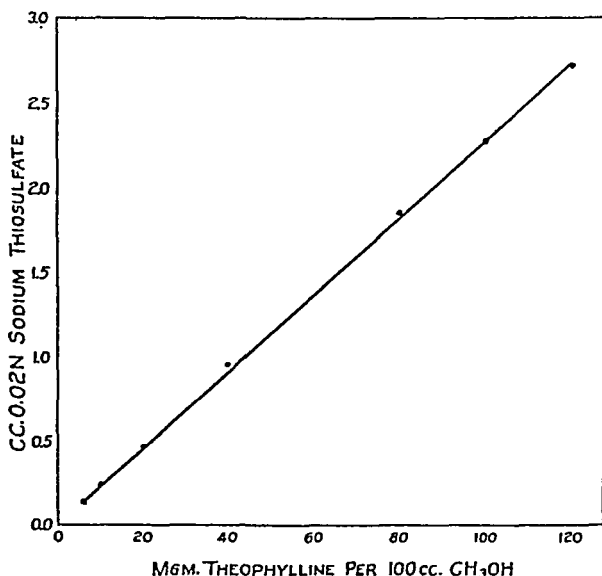


FIG. 1. CONCENTRATIONS OF THEOPHYLLINE AND CUBIC CENTIMETERS OF SODIUM THIOSULFATE

The balance of the determination is carried out as previously described using 0.005 N sodium thiosulfate solution for the final titration. 1 mgm. of theophylline is equivalent to 2.28 cc. of 0.005 N sodium thiosulfate.

Analyses of various amounts of theophylline added to blood in vitro are shown in Table 2.

Application of Method to Urine.

1. Adjust the pH of the urine to between 7.3 and 8.2 by the addition of 2.5 N NaOH and one fifth volume of phosphate buffer of pH 8.0.

2. The balance of the quantitation is carried out as described under the determination in blood, steps 5 through 8. The volume of methyl alcohol used

² This extraction mixture was proposed by Reimers (3) as being more efficient than chloroform alone.

to dissolve the theophylline should be from 1 to 2 cc. 0.01 N sodium thiosulfate should be used for the final titration. 1 mgm. of theophylline is equivalent to 1.14 cc. of 0.01 N sodium thiosulfate.

Analyses of various amounts of theophylline added to urine in vitro are shown in Table 3.

The data on Graph 1 show that between a concentration of 6 and 100 mgm. of theophylline per 100 cc. of methyl alcohol the cc. of thiosulfate bear a direct

TABLE 4

Blood theophylline levels (mgm./100 cc.) after intravenous administration of 10 mgm. per kgm. of theophylline with ethylene diamine

	5 MIN.	30 MIN.	60 MIN.	90 MIN.
Dog 1	0.90	0.54	0.25	0
Dog 2	0.85	0.48	0.20	0
Dog 3	1.10	0.58	0.30	0.13
Dog 4	0.76	0.43	0.20	0
Dog 5	0.92	0.47	0.16	0

TABLE 5

Urinary excretion of theophylline in mgm. after intravenous administration of 10 mgm. per kgm. of theophylline with ethylene diamine

	WT. KG.	0-30 MIN.	30-60 MIN.	60-90 MIN.
Dog 1	10.5	0.52	0.84	0.50
Dog 2	21.0	0.75	0.50	0.32
Dog 3	9.0	0.85	0.45	0.45
Dog 4	7.7	0.60	0.40	0.28
Dog 5	7.0	0.70	0.35	0.44
Average	11.0	0.68	0.51	0.40
Average mgm./Kg.		0.062	0.046	0.036

proportion to the theophylline concentration. In blood and urine determinations theophylline concentrations fall within this range.

Determinations in the Dog.

Theophylline was determined in the blood and urine of five dogs at intervals after the intravenous administration of 10 mgm. per kgm. of theophylline with ethylene diamine. The results are shown in Tables 4 and 5. It is apparent from these data that measurable excretion of theophylline is only 0.14 mgm. per kgm. of theophylline in 90 minutes out of a total of 8 mgm. per kgm. available. This represents an excretion of but 1.8% of the injected theophylline. The method has been applied to human blood and urine with satisfactory results.

It is planned to determine the fate of the balance of the drug in the body by a similar method.

DISCUSSION

15 to 20 cc. samples of blood are satisfactory for ordinary determinations of theophylline. For the detection of amounts of theophylline of the order of 0.13 mgm. per 100 cc. blood it is necessary to use a 25 to 30 cc. blood sample. A dilute urine may be used directly but a concentrated one should be diluted with 3 or 4 volumes of water to facilitate the extraction process.

It is necessary to rigidly exclude water from the solutions when precipitating the theophylline with copper acetate otherwise the precipitation may not be quantitative.

SUMMARY

A method for the quantitative determination of theophylline in blood and urine has been described.

The method is sensitive to 0.13 mgm. anhydrous theophylline per 100 cc. of blood or urine.

Caffeine, theobromine, uric acid, ethylene diamine and sodium acetate do not interfere with the determination nor does any normal blood property or constituent.

After 8 mgm. per kgm. theophylline (in aminophylline) intravenously administered to dogs the theophylline was no longer detected in the blood at the end of 90 minutes in 4 out of 5 dogs.

1.8% of the injected theophylline appears in the urine during this 90 minute period.

REFERENCES

1. TRUITT, E. B., CARR, C. J., BUBERT, H. M., AND KRANTZ, J. C.: *THIS JOURNAL*, **91**: 185 1947.
2. PLUMMER, A. J., AND MENDENHALL, W. L.: *THIS JOURNAL*, **63**: 31, 1938.
3. REIMERS, F.: *Dansk. Tids. Farm.*, **9**: 11, 1935.

THE CHRONIC ORAL TOXICITY OF CHLOROQUINE

O. GARTH FITZHUGH, ARTHUR A. NELSON AND OMA L. HOLLAND

*Division of Pharmacology, Food and Drug Administration, Federal Security Agency,
Washington, D. C.*

Received for publication January 31, 1948

Detailed studies of the antimalarial properties, pharmacology and toxicology of chloroquine (SN-7618), one of the few promising antimalarial drugs among the thousands tested during the past few years (1), have been conducted by numerous investigators and these results are summarized in the recent survey of Wiselogle (2). Since chloroquine has proved more effective in the treatment of vivax malaria than quinacrine (3, 4), most of these studies on the toxicology of chloroquine have compared the toxicities of the two substances. The acute oral toxicity of chloroquine in the rat and monkey is about the same as that of quinacrine. Short-term chronic experiments extending up to 180 days in the rat and monkey indicate that chloroquine is slightly less toxic than quinacrine (2). In the dog chloroquine is more toxic acutely than quinacrine; however, in chronic experiments chloroquine has about the same toxicity as similar dosages of quinacrine (5). The present report extends the preliminary results observed in this laboratory on the chronic oral toxicity of chloroquine to rats and reported by Wiselogle (2) and compares the results of a lifetime study with our published observations on quinacrine (6).

METHOD. Groups of 10 female and 10 male weanling rats (21 days) from our colony of Osborne-Mendel strain were started on each of 6 diets containing respectively 0, 100, 200, 400, 800 and 1000 p.p.m. chloroquine. The basic diet was composed of the following ingredients: dextrose 63%, casein 18%, corn oil 6%, brewer's yeast 5%, salt mixture (U.S.P. XIII No. 2) 4%, whole liver powder 2% and cod liver oil 2%. The chloroquine was mixed with the basic diet by means of a dough mixer. Litter mates were selected and assigned to the various groups according to a randomized design of experiment (balanced incomplete blocks) (7). All animals were kept in individual cages in a room with controlled temperature and humidity and were given free access to their respective diets and water. Body weights and food consumption were determined at weekly intervals. Surviving animals were sacrificed at the end of a two-year period.

RESULTS. *The Effect on Growth and Food Consumption.* The differences in mean gain in weight of the rats during the fast growing period are shown in Table 1. Rats on 100 and 200 p.p.m. chloroquine did not differ significantly ($p = .05$ or less was taken as significant) from the controls. At 400 p.p.m. there was a significant inhibition of growth during the first three months of the experiment. Most of this was made up during the following months, so that by the end of the first year the difference from the controls was no longer significant. At 800 and 1000 p.p.m. there was a marked interference with growth from the outset. These animals remained stunted until their death.

The differences in weekly food consumption between the controls and each

group of experimental animals during the first twelve weeks and during the second twenty-six weeks of the experiment were not significant for the rats on 400 p.p.m. or less chloroquine. The highly toxic dosages of 800 and 1000 p.p.m. chloroquine significantly retarded the food intake.

The Effect on Mortality. All rats on concentrations of 800 and 1000 p.p.m.

TABLE 1
Mean gain in weight of rats fed diets containing chloroquine

TIME ON EXPERIMENT	DOSAGE OF CHLOROQUINE	SEX	NO. OF ANIMALS	MEAN GAIN IN WEIGHT
mos.	p.p.m.			Grams
3	0	M	10	357.3 \pm 8.5
		F	10	216.0 \pm 6.4
3	100	M	10	368.8 \pm 14.7
		F	10	221.1 \pm 10.7
3	200	M	10	337.0 \pm 4.2
		F	10	191.3 \pm 9.1
3	400	M	10	291.7 \pm 17.8*
		F	10	181.0 \pm 8.1*
3	800	M	10	169.9 \pm 9.8†
		F	10	122.5 \pm 7.1†
3	1000	M	10	100.2 \pm 22.1†
		F	10	73.9 \pm 6.3†

* $p < .01$.

† $p < .001$.

TABLE 2
Per cent mortality of rats fed diets containing chloroquine

DOSAGE OF CHLOROQUINE	6 MOS.	12 MOS.	18 MOS.	24 MOS.
p.p.m.				
0	5	15	35	65
100	0	5	45	65
200	5	15	35	70
400	10	15	40	80
800	5	100	100	100
1000	100	100	100	100

chloroquine died within the first year (Table 2). The greater number of the deaths in each of these groups fell within a period of about eight weeks. Most of the rats on 1000 p.p.m. died between the thirteenth and twenty-first weeks (mean survival time 15 weeks) and similarly those on 800 p.p.m. died between the twenty-seventh and thirty-fifth weeks (mean survival time 32 weeks). All

the deaths during the first year in the groups on the lower concentrations of chloroquine were caused either by respiratory infection or by middle ear disease, and on these concentrations no group had more deaths than the control. At the termination of the experiment no group which survived beyond the first few months had a death rate significantly different from that of the control; however, when all groups were considered together there was a significant positive correlation ($r = +.964$; $p = .034$) between the dietary level of chloroquine and the number of survivors.

Hematology. Blood studies were made three times during the first year and once during the second year of the experiment on five or more rats from a group, except in those cases where too few survivors remained in a group to continue the studies. The outstanding change was a leukocytosis, predominantly neutrophilic. It was marked in the group on 800 p.p.m. chloroquine, less striking in the group on 400 p.p.m., and scarcely noticeable in those on 200 p.p.m. A slight increase in hemoglobin concentration and erythrocyte counts was noted in the group on 800 p.p.m. chloroquine.

Pathology. The detailed description of the anatomical lesions caused by the feeding of chloroquine will be published elsewhere (8), and therefore only a brief summary will be given here. At autopsy, the gross lesions varied from marked in degree at the higher dosage levels to minimal at the lowest. At 1000 p.p.m. a majority of the animals had distinctly pale viscera, and at 1000 and 800 p.p.m. there was frequently slight roughening of the liver surface, slight pitting of the kidneys, atrophy of the testes, and a darker than usual color of the pancreas, in fact a general tan tinging of the viscera. From the histopathological standpoint, a relatively great variety of changes was produced in comparison with the feeding of most other compounds. The really lethal lesions were two—a slow focal necrosis of striated muscle, especially cardiac, and secondly, a moderate degree of centrilobular hepatic necrosis and fibrosis.

Other changes, frequent at the higher dosage levels but not necessarily lethal, were testicular atrophy, the presence of foamy macrophages in several locations (lung, intestine, spleen, kidney), pigment in uterine muscle and renal convoluted tubular cells, foamy renal looped tubular cells, and degenerative changes in the pancreatic acinar cells. Generally speaking, there was a sharp drop in incidence and/or severity of lesions between the 800 p.p.m. and the 400 p.p.m. levels. At 1000 and 800 p.p.m., anatomical lesions were marked to very marked, at 400 p.p.m. they were moderate, at 200 p.p.m. slight, and at 100 p.p.m. questionable. A total of 86 rats fed chloroquine plus 15 controls were examined microscopically in detail.

Discussion. The many toxic effects produced by chloroquine in these experiments warrant a consideration of the relationship of the dosage received by the rats and the human dose in the treatment of malaria. The recommended dosage regimens in human malaria have been 300 mgm. given on the same day each week for suppression and 600 mgm. given as an initial dose followed by an additional 300 mgm. after 6 to 8 hours and a single dose of 300 mgm. on each of the next two days for the treatment of an acute attack of vivax malaria (1). Chart 1 shows

the calculated chloroquine intake per day in terms of the body weight of the rat. These values were calculated from the weekly food intake of the rats at the various monthly intervals. The chloroquine intake of the rats at any given dosage level decreased rapidly during the first two months and then became almost constant as shown by the straight line (chart 1) at about six months on the experiment diet. This fact is accounted for by the change in the growth rate of the rats from the fast growing period to the plateau period, while at the same time their daily food intake remained almost constant. The slight difference in dosage per kgm. of body weight between the female and male animals

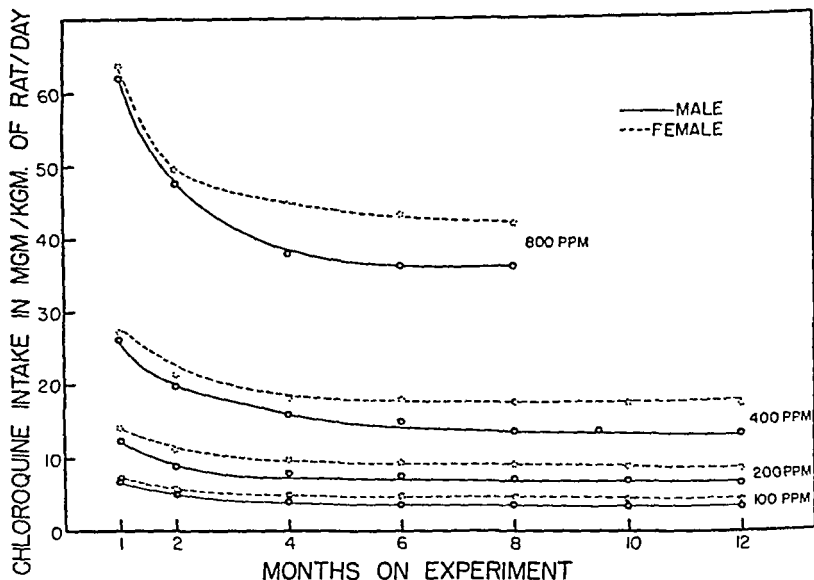


Chart I. Calculated chloroquine intake in MGM/KGM of body weight in rats receiving various levels of chloroquine in the diet.

was produced by the slightly greater amount of food consumed by the females in relation to their body weight than by the male rats. This increased chloroquine intake did not produce any demonstrable difference in toxicity between the sexes fed similar concentrations. As shown in the chart the dosage of 100 p.p.m. chloroquine corresponds to about 4 mgm./kgm. of body weight per day for the rat over the greater part of the two-year experiment. This dosage level for the rat was a borderline at which slight or questionable damage occurred to some rats given chloroquine for two years and no harm occurred to others. It corresponds approximately to the prophylactic dosage in man; however, taking the length of time into consideration, the amount of chloroquine that will produce toxic effects in rats is above the therapeutic or prophylactic dose for man.

Considering the entire series, it is our impression that the toxicity of chloro-

quine is slightly less than that of quinacrine to rats. The histopathological lesions observed in these experiments showed considerable similarity to those following similar dosages of quinacrine. The chief difference was in the lack of massive hepatic necrosis with chloroquine; muscle lesions were, however, somewhat greater. On the same diet quinacrine produced a slightly greater effect on mortality at the 400 p.p.m. concentration. All animals on this dosage level of quinacrine died while some animals on the same amount of chloroquine survived the full two-year experimental period. Likewise quinacrine had a slightly greater effect on growth at the concentration of 200 p.p.m. There was no difference in the effect of the two substances on rats at the low dosage level of 100 p.p.m. in the diet. In general, the 400 p.p.m. dosage level of both substances showed a marked drop in severity of lesions as compared with higher levels.

SUMMARY

1. A two-year chronic toxicity study with rats fed diets containing from 100 to 1000 p.p.m. chloroquine showed that the toxicity of chloroquine was very slight or questionable at 100 p.p.m. and became progressively more severe with each increase in dosage.

2. There was a significant retardation of growth at a concentration of 400 p.p.m.; lower dosages produced no effect on growth.

3. A progressive increase in mortality occurred at dosage levels of 200 p.p.m. or more; the 800 and 1000 p.p.m. chloroquine caused early death of all animals.

4. The outstanding hematological change was a leukocytosis, predominantly neutrophilic. This was marked in the group on 800 p.p.m., less striking in the group on 400 p.p.m. and scarcely noticeable in those on 200 p.p.m. chloroquine. There was an increase in the hemoglobin concentration and erythrocyte counts in the rats on the 800 p.p.m. chloroquine.

5. Histopathological changes increased from very slight or absent in rats on 100 p.p.m. to marked in those on 800 and 1000 p.p.m. The two prominent lesions at toxic dosages of chloroquine were a slow focal necrosis of striated muscle, especially cardiac, and a moderate degree of centrilobular hepatic necrosis and fibrosis.

6. In relation to body weight of the rat the lowest dosage of chloroquine which produced slight toxic effects in some animals corresponds to approximately 4 mgm./kgm./day for two years.

7. In comparison with quinacrine fed to rats at similar dosage levels and with a similar diet, the anatomical changes produced by chloroquine were similar; however, the toxicity of chloroquine was, on the whole, slightly less than that of quinacrine. At the low dosage level of 4 mgm./kgm./day there was no noticeable difference between the toxicities of the two substances.

REFERENCES

1. LOEB, R. F. ET AL. Statement approval by the Board for Coordination of Medical Studies, J. A. M. A., 130: 1069, 1946

2. WISELOGLE, F. Y.: Survey of Antimalarial Drugs 1941-1945, J. W. Edwards, Ann Arbor, Michigan, 1946.
3. MOST, H., LONDON, I. M., KANE, C. A., LAVIETES, P. H., SCHROEDER, E. F., AND HAYMAN, J. M., JR.: J. A. M. A., 131: 963, 1946.
4. MARSHALL, E. K., JR.: Fed. Proc., 5: 298, 1946.
5. Unpublished observations from this laboratory.
6. FITZHUGH, O. G., NELSON, A. A., AND CALVERY, H. O.: THIS JOURNAL, 85: 207, 1945.
7. FISHER, R. A., AND YATES, F.: Statistical Tables for Biological, Agricultural, and Medical Research, Oliver and Boyd, Edinburgh, 1943.
8. NELSON, A. A., AND FITZHUGH, O. G.: Arch. Path., in press.

EFFECT OF VITAMIN A DEFICIENCY ON THE TOXICITY OF STRYCHNINE

JOHN F. SUCHY AND CHARLES F. POE*

Division of Sanitary Chemistry, University of Colorado, Boulder, Colorado

Received for publication February 3, 1948

In a previous publication (1) the toxicity of strychnine was reported for male and female rats of different ages. The lethal dose for 50 per cent of the animals (L.D.₅₀) increased with male rats from 1.4 mgm. per kilogram for those 6 weeks of age to 2.3 mgm. per kilogram for rats 6 months old. The range for female rats of corresponding ages increased from 0.9 mgm. to 1.4 mgm. per kilogram. About the time this investigation was under way, there were available in the laboratory a large number of white rats that had undergone a rather severe vitamin A deficiency. The animals which had shown an actual loss in weight from 20 to 45 grams during an experimental period of at least 10 weeks on a vitamin A deficient diet were selected. These deficient animals were placed on a normal diet for a period of three weeks before being injected with strychnine. Therefore, the results obtained are on the basis of the permanent effects of vitamin A deficiency.

The references in the literature concerning the effect of vitamin deficiency on the toxicity of strychnine are not conclusive. Smith, McClosky, and Hendrick (2) report a slight increase in susceptibility of vitamin A-deficient rats to strychnine poisoning. The M.L.D. for normal mature rats was 0.9 mgm. per kilogram; whereas the M.L.D. for vitamin A-deficient rats was 0.7 mgm. per kilogram. These experiments were based on seven normal animals and nine deficient animals. No mention was made of the sex of the animals. Saiki (3), on the other hand, states that white rats deficient in Vitamin A can stand larger doses of strychnine than animals fed a normal diet.

A pure sample of strychnine sulfate was selected and the same sample was used throughout the experiment. This precaution was necessary because Ward, Munch, and Garlough (4) and Poe, Suchy, and Witt (1) report a slight variation in toxicity of different samples of strychnine.

The strychnine sulfate was dissolved in physiological salt solution so that each ml. of solution contained the equivalent of 1 mgm. of strychnine alkaloid. Fresh solutions were prepared each day that injections were made. The doses were administered by intraperitoneal injections. The animals were placed in individual cages, care being taken to prevent undue excitation of the animals during the course of the experiment. Records of the time to convulsions and death were carefully made. Albino rats, progeny of the Wistar strain, were used throughout the entire experiment. These animals were raised in our laboratory under carefully controlled conditions. The animals at the time of injection were between 17 and 18 weeks of age, and were of the same weight group.

Because of individual resistances of some of the animals to strychnine, a large number of animals should be used on each dosage in order to determine more accurately the lethal dose. Over 1200 deficient rats and around 1000 normal ones were used.

* For reprints address the junior author, University of Colorado, Boulder, Colorado.

The toxicity is expressed in the form of curves as proposed by Trevan (5). The toxicity in any given instance may be found by selecting the point where the curve crosses the 50 per cent mortality line, and then dropping a perpendicular to the dosage line. The curves for normal and vitamin A-deficient animals are shown in fig. 1. The toxicities for male and female rats are shown in separate curves.

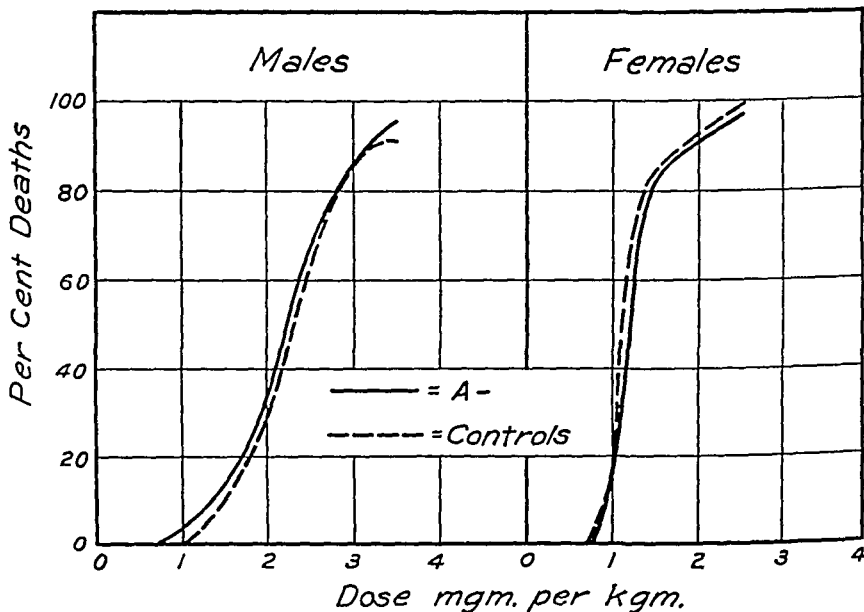


FIG. 1. TOXICITY CURVES FOR VITAMIN A DEFICIENT AND CONTROL RATS

From the curves the lethal dose of strychnine for 50 per cent of the male rats is as follows: control rats 2.27 mgm. per kilogram, deficient rats 2.23 mgm. per kilogram. The corresponding values for female rats are 1.20 mgm. and 1.19 mgm. The previous values for normal rats 18 weeks of age reported from this laboratory are: male rats, 2.3 mgm. and female rats, 1.1 mgm. per kilogram (1).

With male rats the average times to convulsions for dosages of 1 and 4 mgm. per kilogram were 9.6 and 3.0 minutes, respectively. The average times to death for the same dosages were 10.4 and 4.8 minutes, respectively. The values for female rats were of about the same order.

CONCLUSIONS

1. The toxicities of strychnine for normal and vitamin A-deficient white rats vary but little.

2. Confirmation of previous work in this laboratory shows strychnine to be more toxic for female rats than for male rats.

REFERENCES

1. POE, C. F., SUCHY, J. F., AND WITT, N. F.: *THIS JOURNAL*, 58: 239, 1936.
2. SMITH, M. I., McCLOSKEY, W. T., AND HENDRICK, E. G.: *U. S. Pub. Health Reports*, No. 41, pp. 767, 1926.
3. SAIKI, S.: *Bull. Soc. Hyg. Aliment.*, 15: 481, 524, 1927. See *C. A.*, 22: 2775, 1928.
4. WARD, J. C., MUNCH, J. C., AND GARLOUGH, F. E.: *Jour. Am. Phar. Assoc.*, 25: 59, 1936.
5. TREVAN, J. W.: *Proc. Roy. Soc. (London)*, 101: 483, 1927.

OUABAGENIN

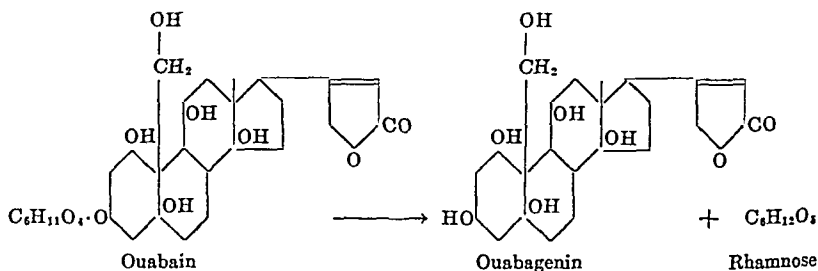
K. K. CHEN, ROBERT C. ANDERSON, AND HAROLD M. WORTH

The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis 6, Indiana

Received for publication February 9, 1948.

Because of the unstable nature of ouabagenin, the aglycone of ouabain or G-strophanthin, as shown by Jacobs and Bigelow (1), no pure sample has heretofore been obtained by the usual methods of degradation for the establishment of its chemical structure and the determination of its pharmacologic activity. On the other hand, certain suggestions of a structural formula for ouabain and thus, also, for ouabagenin, have been made by Fieser and Newman (2), Tschesche and Haupt (3), and Marker and associates (4).

Mannich and Siewert (5), whose work Reichstein and Reich (6) recently reviewed, developed a method of mild hydrolysis, and succeeded for the first time in isolating from the reaction ouabagenin in crystalline form. Their results culminated in the proposal of a new formula for ouabain, which, in its simplest term, will give rise to ouabagenin and the sugar rhamnose as follows:



Professor T. Reichstein, director of the Pharmaceutical Institute of the University of Basel, Switzerland, to whom we are greatly indebted, prepared especially for our pharmacologic investigation a batch of ouabagenin. The melting point of the fresh sample was 263° to 265°C ., but dropped to about 250°C . upon standing, probably due to the formation of a hydrate (6). The product was analytically pure.

For pharmacologic experiments, a stock solution of ouabagenin, 0.1%, was prepared in 47.5% ethanol, and subsequently diluted in saline to suitable concentrations. The typical digitalis-like action of ouabagenin was quickly demonstrated as evidenced by the occurrence of systolic ventricular standstill in frogs, and of bradycardia, arrhythmia, secondary tachycardia, and terminal ventricular fibrillation in cats. We then proceeded to determine its exact potency in 11 etherized cats by the intravenous injection of a 1:50,000 solution at the rate of 1 cc. per minute. It was also assayed in frogs by the 1-hour method with 1:10,000 and 1:2,000 concentrations, injected into the ventral lymph sac. In addition, the emetic action of ouabagenin was tested out in non-anesthetized cats by rapid intravenous injection of the stock solution.

The results in anesthetized cats are listed in table 1. The mean¹ lethal dose (LD) of 11 animals is 238.9 $\mu\text{g.}$ per kg. computed geometrically. Compared with its parent glycoside ouabain, which has a mean LD of 116 $\mu\text{g.}$ per kg. (7), ouabagenin is approximately one-half as potent. The mean LD of ouabain was computed from the protocols of a previous paper (8). It illustrates very definitely that the presence of a sugar on C_3 , which in this case is rhamnose, enhances the cardiac activity, thus making ouabain twice as strong as ouabagenin. This is in line with the results of 4 other pairs of aglycones and their glycosides previously reported from this laboratory, namely, strophanthidin, periplogenin, digoxigenin, digitoxigenin, cymarín, periplocymarín, digoxin, and digitoxin, respectively (7-11). They are contrasted in figure 1, all expressed in the number of mean LD's in 1 mg. The difference in activity between an aglycone and its parent glycoside may be even greater if the reaction of hydrolysis causes an alteration of

TABLE 1
Determination of Lethal Dose in Etherized Cats

CAT NUMBER	SEX	BODY WEIGHT	DOSE TO KILL
		kg.	$\mu\text{g. per kg}$
1	F	2.778	165.5
2	F	2.555	254.2
3	F	2.542	360.3
4	M	2.245	360.8
5	F	2.480	171.0
6	F	2.600	158.8
7	F	2.563	241.9
8	M	2.505	169.3
9	M	2.415	229.4
10	F	1.998	338.3
11	M	1.951	305.5
Mean LD \pm standard error .			238.9 \pm 23.4

the aglycone molecule. This is exemplified by scillaridin A and scillaren A, and calotropagenin and calotropin, respectively (10).

In frogs, the number of systolic hearts to the number of animals used, with various doses, were as follows: 1/10 with 1.5, 5/10 with 2, 7/10 with 2.5, 9/10 with 3.5, and 5/5 with 5, $\mu\text{g. per g.}$ The median systolic dose \pm standard error is thus $2.127 \pm 0.166 \mu\text{g. per g.}$ Assayed side by side, ouabain showed the following: 0/10 with 0.5, 4/10 with 0.7, and 8/10 with 0.9, $\mu\text{g. per g.}$; the median systolic dose \pm standard error being $0.7513 \pm 0.041 \mu\text{g. per g.}$ The simultaneous standardization of the 2 compounds, in the same batch of animals, was necessary, because frogs, unlike cats, do not easily repeat results. Unless studied at the same time and in the same batch of frogs, the figures for 2 digitalis-like products are

¹ According to statistical principles, the geometric mean is preferable to the arithmetical mean. The former is the antilogarithm of the mean of logarithms of individual doses.

best not used for comparison. In the present series of experiments in frogs ouabain is 2.8 times as potent as ouabagenin. This is probably as close an agreement as may occur between the cat and the frog methods. Occasionally, the order of activity by the 2 methods is just reversed. For example, cymarol is more potent than cymarin in cats, but weaker than cymarin in frogs (12).

The emetic action of ouabagenin in non-anesthetized cats is not only preserved, but also unimpaired. The number of animals that vomited with various doses, to the number of animals used, were as follows: 1/3 with 36.5, 2/3 with 50, and 1/1 with 70, $\mu\text{g. per kg.}$ The median emetic dose \pm standard error ($\text{EmD}_{50} \pm \text{S. E.}$) is therefore $42.53 \pm 5.95 \mu\text{g. per kg.}$ Ouabain studied previously and calculated in a similar manner, having an $\text{EmD}_{50} \pm \text{S. E.}$ of $58.4 \pm 9.0 \mu\text{g. per kg.}$, is actually less active than ouabagenin, weight for weight. This is also true with digitoxin and digoxin, although on the heart they are more potent (10). The vomiting dose of periplocymarin was already determined (9), the $\text{EmD}_{50} \pm \text{S. E.}$ by calculation being $75 \pm 16.5 \mu\text{g. per kg.}$ In the present study, periploge-

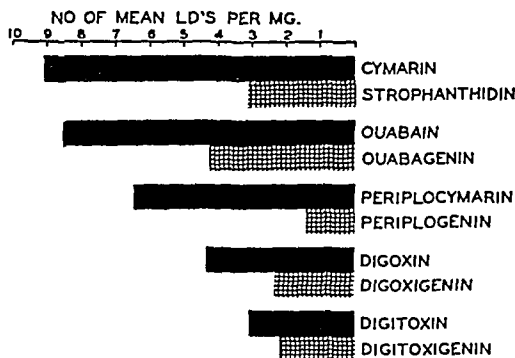


FIG. 1. COMPARISON OF CARDIAC ACTIVITY OF 5 PAIRS OF AGLYCONES AND THEIR GLYCOSIDES

nin in 1:1,000 solution with 47.5% ethanol was injected into 8 non-anesthetized cats. The frequency of vomiting was in the following order: 0/1 on 50, 1/3 on 70, 2/3 on 100, and 1/1 on 200, $\mu\text{g. per kg.}$ The $\text{EmD}_{50} \pm \text{S. E.}$ is $85.6 \pm 15.4 \mu\text{g. per kg.}$ By calculation of our published results (10), the EmD_{50} 's of cymarin and strophanthidin are 74.8 ± 15 and $82.7 \pm 7 \mu\text{g. per kg.}$, respectively. Proportionately, periplogenin and strophanthidin have a higher emetic activity than their parent glycosides, periplocymarin and cymarin, as compared with their cardiac activity. This becomes very obvious in figure 2 when the ratios of mean LD_{50} 's to EmD_{50} 's of 5 pairs of compounds are computed.

It must be understood that the EmD_{50} was determined by rapid intravenous injection in non-anesthetized cats, and the mean lethal dose, by slow infusion of a suitable solution from the femoral vein of etherized cats. The rate of injection in this case was 1 cc. per minute, so that the animal died within 30-60 minutes. The two sets of results, therefore, may not be comparable. The problem becomes further complicated when one realizes that aglycones are generally more rapidly

eliminated than their parent glycosides. Even given at the same rate of slow infusion to cause death at the same time, more aglycones may be required due to their rapid elimination. Any observed difference in such instances may therefore only be apparent and not real.

To test this point, ouabain and ouabagenin were adopted as examples, and were injected in a group of 15 non-anesthetized cats—8 with the former, and 7 with the latter. Both drugs were in a 1:2,000 solution with 23.7% ethanol. All injections were made intravenously and rapidly—in a fraction of a second. The results in general were confirmatory of those by slow injection. Ouabain in the dose of 160–225 $\mu\text{g. per kg.}$ killed 3 cats in from 6–28 minutes. One animal died in 6 hours on a dose of 140 $\mu\text{g. per kg.}$, and 3 others lived on doses from 70–110 $\mu\text{g. per kg.}$ Ouabagenin in doses varying from 330–400 $\mu\text{g. per kg.}$ killed 3 cats in 4–10 minutes. The remaining 4 animals survived on smaller doses. It thus

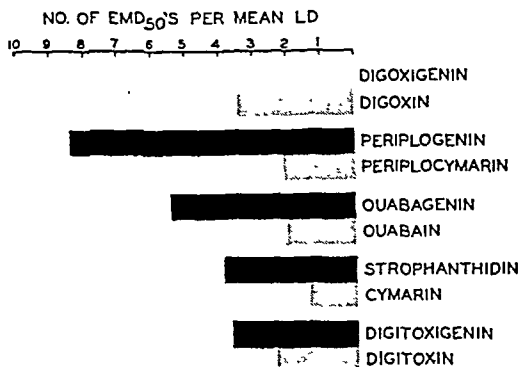


FIG. 2. COMPARISON OF EMETIC ACTIVITY OF THE SAME 5 PAIRS OF AGLYCONES AND GLYCOSIDES

appears that even though the time of injection is reduced to less than a second, ouabain still remains a little more than twice as potent as ouabagenin. The difference in activity between this pair must therefore be accepted as real.

In our previous investigation of aglycones, digitoxigenin was shown to cause convulsions in both frogs and cats (10). In connection with the frog assay and the cat-vomiting test of the present work, special attention was paid to this phase of action with ouabagenin. No convulsions occurred. Similarly, periplogenin did not exhibit any convulsant effect in cats.

DISCUSSION

The enhancing effect of the sugar portion of the glycoside molecule can be demonstrated by one of two ways. First, the splitting of the sugar, either a monosaccharide or a polysaccharide, from the parent glycoside by hydrolysis results in reduction of cardiac activity. The decrease in potency from ouabain to ouabagenin is a clear-cut example. Secondly, partial synthesis of a new glyco-

side from an aglycone gives rise to a more active compound as proved with digitoxigenin, digoxigenin, strophanthidin, and periplogenin (11). It may be postulated, therefore, that by coupling a new sugar for rhamnose with ouabagenin a highly potent product may be obtained. Just why a carbohydrate having no action on the heart itself increases the cardiac activity of an aglycone, following a chemical union, can only be left for future speculation or explanation when our knowledge on the site of action of the digitalis group further advances.

The emetic action of ouabagenin, on the other hand, does not diminish as compared with ouabain. The slightly higher activity of ouabagenin to cause vomiting may be due to its smaller molecular size. This is also true with digitoxigenin and digoxigenin, and relatively so with strophanthidin and periplogenin. It thus indicates that while the removal of the sugar radical from a glycoside reduces the cardiac activity, it does not depreciate the emetic activity. Here is the best evidence that the emetic activity is no measure of the cardiac activity. For comparative purposes, they must be considered separately.

SUMMARY

Ouabagenin is approximately one-half as active on the heart as ouabain in cats, and about one-third as active in frogs.

Ouabagenin is more effective than ouabain in causing vomiting of non-anesthetized cats, weight for weight. The emetic dose is therefore no measure of the cardiac activity when different compounds are compared.

Unlike digitoxigenin, ouabagenin and periplogenin do not cause convulsions in cats or frogs.

ACKNOWLEDGEMENT. The authors are indebted to Misses Nila Maze, Marian H. Ellaby, and Messrs. John S. Welles, E. Brown Robbins, Chester C. Hargreaves, and William T. Winchester.

REFERENCES

1. JACOBS, W. A., AND BIGELOW, N. M.: *J. Biol. Chem.*, **96**: 647, 1932; *J. Biol. Chem.*, **101**: 15, 1933.
2. FIESER, L. F., AND NEWMAN, M. S.: *J. Biol. Chem.*, **114**: 705, 1936.
3. TSCHIESCHKE, R., AND HAUPT, W.: *Ber. deut. Chem. Gesellsch.*, **70**: 43, 1937.
4. MARKER, R. E., TURNER, D. L., OAKWOOD, T. S., ROHRMANN, E., AND ULSHAFFER, P. R.: *J. Am. Chem. Soc.*, **64**: 720, 1942.
5. MANNICH, C., AND SIEWERT, G.: *Ber. deut. Chem. Gesellsch.*, **75**: 737, 1942; *Ber. deut. Chem. Gesellsch.*, **75**: 750, 1912.
6. REICHSTEIN, T.: Personal communication.
7. CHEN, K. K.: *Ann. Rev. Physiol.*, **7**: 677, 1945.
8. CHEN, K. K., CHEN, A. L., AND ANDERSON, R. C.: *J. Am. Pharm. Assoc.*, **26**: 579, 1936.
9. CHEN, K. K., ANDERSON, R. C., AND ROBBINS, E. B.: *J. Am. Pharm. Assoc.*, **27**: 113, 1938.
10. CHEN, K. K., ROBBINS, E. B., AND WORTH, H.: *J. Am. Pharm. Assoc.*, **27**: 189, 1938.
11. CHEN, K. K., ELDERFIELD, R. C., UHLE, F. C., AND FRIED, J.: *THIS JOURNAL*, **77**: 401, 1943.
12. CHEN, K. K., AND ANDERSON, R. C.: *THIS JOURNAL*, **90**: 271, 1947.

THE EFFECT OF ADRENALECTOMY ON MORPHINE ANALGESIA IN RATS¹

FRANCES J. FRIEND, M.S., AND S. C. HARRIS, Ph.D.

Department of Physiology, Northwestern University Medical School, Chicago, Illinois

Received for publication February 12, 1948

It has recently been suggested (1) that morphine analgesia is mediated wholly or in part by the release of epinephrine from the adrenal medulla. This hypothesis is based on the evidence that morphine causes the release of epinephrine from the adrenal medulla (2, 3, 4) and more recent reports that epinephrine exerts an analgesic effect (5). This hypothesis implies that the analgesic effect of morphine should be reduced or abolished by removal of the adrenal medulla. It was the purpose of this study to investigate this possibility in rats, and at the same time to determine the statistical reliability of the simple analgesimetric method employed by Eddy (6) and Molitor and Latven (7).

METHOD. The method employed for the determination of "pain" or reaction threshold in rats consisted of applying gradual pressure to the rat's tail by means of a calibrated forceps and recording the diameter of the tail at the moment when the animal reacts by squeaking.

Twenty-four male albino rats were used in this study. Reaction thresholds were determined by the above method three times at 10-minute intervals. A standard dose of 5 mg./kilogram body weight of morphine sulfate was then administered subcutaneously and single reaction threshold determinations were made at 15-minute intervals for the following 2½ hours. This entire procedure was repeated on 5 occasions at 1- or 2-day intervals.

The 24 rats were then divided into 2 groups. One group of 11 animals was bilaterally adrenalectomized and a small portion of the cortical tissue was transplanted into the anterior chamber of the eye according to the method described by Turner (8). The remaining 13 animals were subjected to laparotomy and exposure, but not removal, of the adrenal glands. These rats served as "sham-operated" controls. The above procedure for determining changes in the reaction threshold following morphine administration was then repeated exactly on 5 occasions at 1- or 2-day intervals.

The animals were maintained throughout the experiment on a diet of Wayne Dog Food Blox and lettuce. For one week following operation all rats were given 1.0 per cent sodium chloride for drinking water which has been shown by Anderson (9) to be a dietary necessity in completely adrenalectomized rats. Operated animals were tested approximately three weeks following surgery.

RESULTS. 1. *Reliability of the methods.* There are two aspects of the reliability of the methods employed which require analysis. These include the reliability of the reaction threshold determination, expressed as millimeters of tail diameter at the moment of reaction to pain, and the reliability of the morphine analgesia response, expressed as change in the reaction threshold.

a. *Reliability of reaction threshold determinations.*

Each day, prior to morphine administration, 3 threshold determinations were made on each animal. The mean values of the 24 rats for these three trials

¹ Aided by grant from Smith, Kline, and French Laboratories, Philadelphia, Pa.

on the 5 test days, prior to operation, are presented in Table 1. A preliminary analysis of variance (10) on these data revealed highly significant differences between days with significant interaction between rats and days. Inspection of the data (Table 1) suggested that these significant differences between days may be due to the low values obtained on day 1. When the values of that day were omitted, the highly significant differences between days were eliminated. Therefore the analysis of variance given in Table 1 evaluated data from days 2 to 5 only.

This analysis reveals that the reliability of a single reaction threshold determination was high as shown by the intraclass correlation of 0.97 and the highly significant differences between individual rats. The reliability signifies that

TABLE 1

Reliability of the reaction threshold determination; mean pain threshold of 24 normal rats

TRIALS	TEST DAYS					MEAN
	1	2	3	4	5	
1	7.49	8.18	8.17	8.20	8.29	8.06
2	7.49	8.24	8.16	8.25	8.25	8.08
3	7.50	8.27	8.13	8.20	8.28	8.08
Mean	7.49	8.23	8.15	8.21	8.27	8.07

Analysis of Variance

SOURCE OF VARIATION	DEGREES FREEDOM	MEAN SQUARE	F RATIO
Differences between rats	23	2.003	34.53*
Differences between test days	3	.176	3.034†
Differences between trials	2	.010	.172
Error	259	.058	

Interclass Correlation, Coefficient = 0.97.

* Significant at the 1% level.

† Significant at the 5% level.

these differences between thresholds for individual rats are consistent upon repetition of the test.

The analysis further reveals that the three successive trials made each day were not significantly different, indicating that no conditioning, either positive or negative, occurred. There are, however, differences of slight significance (5 per cent level) between the mean values on the 4 different test days. This may be due to the fact that, although the position on the rat's tail where pressure was applied was kept constant on each test day, there was some variation between test days due to the development of a sore or swelling. This factor also appears to be responsible for the lowered intraclass correlation coefficient of 0.91 which was obtained when the data (Table 1) were further analyzed by comparing the means of 3 trials for each rat on days 2 to 5. This, however, is to be expected, since there are greater opportunities for variation both in the technique of

measurement and in the characteristic being measured over a period of days than over a period of minutes. Nevertheless, the reliability coefficient of 0.91 is satisfactorily high.

Although the measurement has a high reliability, on *a priori* grounds its validity as a measure of pain threshold is questionable, since the tail diameter at the moment of pain reaction must be influenced not only by pain sensitivity, but also by the anatomical diameter of the tail. The anatomical component in this measurement undoubtedly contributes significantly to the reliability while impairing the validity. This defect in validity could undoubtedly be largely overcome by modifying the forceps to register pressure rather than diameter.

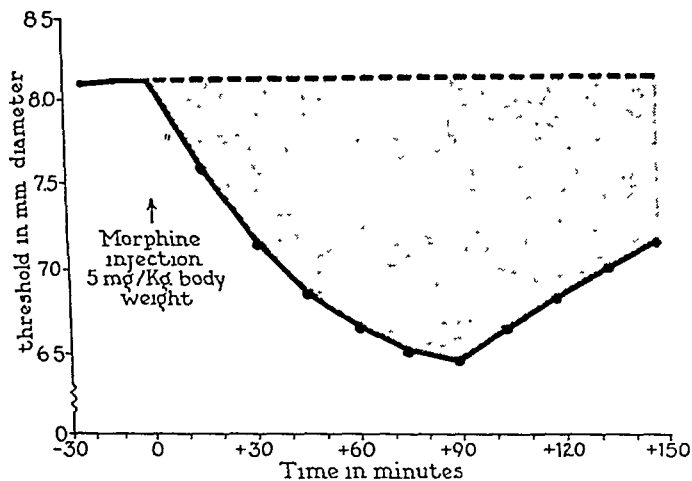


FIG. 1. ANALGESIC RESPONSE TO MORPHINE; MEAN OF 5 TESTS ON EACH OF 24 NORMAL RATS

b. Reliability of the measurement of analgesic response.

On each test day after 3 pre-morphine threshold* determinations had been made, morphine was injected subcutaneously in a dose of 5 mg./kilogram body weight, and single threshold determinations were made at 15-minute intervals for the following 2½ hours. The mean of all such threshold curves obtained before operation is presented in figure 1, which reveals the typical duration-effect curve with a maximum effect at 90 minutes.

In order to provide a single figure to represent the entire duration-effect curve, and in order to remove insofar as possible the non-valid anatomical component of the reaction threshold measurement, each individual morphine response curve was handled as follows. From each of the 10 post-morphine measurements the mean of the 3 pre-morphine trials was subtracted. The resulting 10 decrements in tail diameter were then added together and the sign changed from negative to positive. The resulting value was taken as the *measure of analgesic response*; it is represented by the shaded area of figure 1.

TABLE 2
Reliability of the analgesic response to morphine

MEAN ANALGESIC RESPONSE	TEST DAY					DIFFERENCES BETWEEN RATS		DIFFERENCES BETWEEN DAYS		FREER	RELIABILITY COEFFICIENT (INTRAClass CORRELATION)
	1	2	3	4	5	Mean Square	F Ratio	Mean Square	F Ratio	Mean Square	
24 normal rats	11.9	13.9	12.7	12.1	13.0	19.45	2.17*	13.98	1.56	8.94	0.68
11 adrenalectomized rats ..	7.8	9.2	8.2	7.2	8.1	10.20	2.84*	6.10	1.70	3.58	0.74
13 laparotomized rats..	9.9	12.3	11.0	11.8	11.2	25.06	5.37*	9.63	2.06	4.66	0.84

* Significant at the 1% level.

TABLE 3
Analgesic response of rats to morphine before and after adrenalectomy or laparotomy

RAT NO.	MEAN OF 5 TESTS BEFORE OPERATION	MEAN OF 5 TESTS FOLLOWING ADRENALECTOMY	DIFFERENCE BETWEEN MEANS	RAT NO.	MEAN OF 5 TESTS BEFORE OPERATION	MEAN OF 5 TESTS FOLLOWING LAPAROTOMY	DIFFERENCE BETWEEN MEANS
1	12.86	9.38	3.48	12	12.32	10.80	1.52
2	15.62	7.06	8.56	13	16.74	15.02	1.72
3	13.58	10.64	2.94	14	12.45	13.82	-1.37
4	14.90	7.26	7.64	15	13.58	14.32	-.74
5	14.94	8.34	6.60	16	11.66	10.94	.72
6	15.86	8.76	7.10	17	11.02	11.34	-.32
7	10.96	7.50	3.46	18	10.34	9.86	.48
8	9.82	5.68	4.14	19	10.34	10.26	.08
9	11.70	9.72	1.98	20	13.98	10.48	3.50
10	12.72	7.02	5.70	21	12.04	8.56	3.48
11	13.70	7.94	5.76	22	10.40	7.50	2.90
				23	12.66	12.44	.22
				24	10.77	12.42	-1.65
Mean ..	13.33	8.11	5.21		12.17	11.36	.810
Standard Deviation	±1.82	±1.42	±2.14		±1.82	±2.19	±1.71
Standard Error			±.644				±.475
T Ratio			8.09				1.70
Signif. .			High				None

Table 2 presents the results of the statistical analysis on the measurements of analgesic response in the normal, laparotomized and adrenalectomized rats. The reliability coefficient based on the 5 repeated tests in 24 rats before opera-

tion was found to be 0.68, which is significant as shown by the significant differences between rats. This coefficient is appreciably lower than the previous ones discussed, principally because of variation in response to morphine and because the anatomical component of the reliability has been largely removed.

Table 2 also reveals that the 5 successive morphine analgesia responses did not differ significantly from one another. There was, therefore, neither conditioning nor development of morphine tolerance. Similar conclusions apply to the adrenalectomized and laparotomized animals.

2. *The effect of adrenalectomy on the analgesic response to morphine.*

The means of 5 determinations of the analgesic response to morphine for all animals both before and after adrenalectomy or laparotomy are presented in Table 3. The mean analgesic response of the 11 animals before adrenalectomy was 13.33, whereas after operation it was reduced to 8.11. This reduction, when analyzed by the method of paired comparison, proved to be highly significant.

On the other hand, the mean analgesic response of 13 animals was reduced from a control value of 12.17 to 11.36 following laparotomy. This small difference when similarly analyzed proved to have no significance. Thus, the effect of adrenalectomy could not be attributed to the non-specific effect of the operative trauma.

An analysis by the method of group comparison showed that the two series of animals pre-operatively had mean analgesic responses which were not significantly different from one another, but that following operation the adrenalectomized group had a significantly lower analgesic response than the laparotomized animals.

These results, therefore, demonstrate that adrenalectomy reduced the analgesic response to morphine.

DISCUSSION

The results obtained demonstrated that the analgesic response to morphine as revealed by the simple method employed was significantly reduced in adrenalectomized rats with cortical transplants. There are three factors, however, which must be considered in evaluating the significance of these findings. *First*, the analgesimetric device measured tail diameter which is a curvilinear function of the pressure produced. Hence it is of interest to know whether the surgical procedures induced any changes in anatomical tail diameter. Statistical analysis of the pre-medication tail diameters on pain thresholds, revealed no significant changes following adrenalectomy or laparotomy. *Second*, since the morphine was administered on a per kilo basis, it is important to know whether changes in body weight and therefore changes in absolute morphine dosage, could account for the results. The mean body weight of the adrenalectomized animals was 401 grams before and 390 grams after operation; this slight loss of weight was accompanied by the marked reduction in analgesic response to morphine. However, the body weight of the laparotomized animals increased from 386 grams to 406 grams, accompanied by an insignificant reduction in morphine sensitivity.

Changes in absolute dose of morphine, therefore, cannot account for the results. *Finally*, it is important to know whether the adrenalectomized animals suffered only medullary deficiency, or cortical deficiency as well. The fact that they lost only 2.7 per cent body weight during a five-week post-operative period, during only the first week of which they received one per cent sodium chloride as drinking water, indicates that the cortical transplants provided adequate cortical hormone. Puharich and Goetzl (11) have recently reported results similar to ours, and further demonstrated that cortical extracts were without influence on the analgesic effect of morphine.

These results, therefore, indicate that removal of the adrenal medulla reduces, but does not abolish, the analgesic response of rats to morphine. Furthermore, these results are in harmony with the hypothesis that epinephrine released from the adrenal medulla in response to morphine is responsible in part for the analgesic effect of this drug. However, it must be pointed out that complete acceptance of this hypothesis must await additional important evidence. Most conspicuous is the requirement that the quantities of epinephrine actually released in response to morphine must be capable of inducing the required augmentation of the analgesia.

SUMMARY AND CONCLUSIONS

1. The "pain" or reaction threshold of rats was determined by applying pressure to the tail with a special forceps which indicated the diameter of the tail at the time of vocal reaction. This measurement had a very high reliability, or reproducibility, due in part to individual differences in anatomical tail diameter rather than differences in actual pain sensitivity.

2. The analgesic response to 5 mg./kilogram of morphine was determined in rats by measuring the mean change in "pain threshold" during a 150-minute period following subcutaneous injection of the drug. This procedure eliminated most of the effect of anatomical tail diameter, and therefore had lower, but satisfactory, reliability.

3. The analgesic response to morphine of 11 rats was determined on 5 alternate days before, and 5 alternate days after removal of the adrenal glands and transplantation of the cortex to the eye. The mean analgesic response (no tolerance developed) was markedly and significantly reduced. In a parallel experiment in 13 rats in which a sham operation was performed, no significant change in analgesic response was obtained.

4. These results are consistent with, but do not prove, the hypothesis that the release of epinephrine from the adrenal medulla mediates in part the analgesic effect of morphine.

REFERENCES

1. IVY, A. C., GOETZL, F. R. AND BURRILL, D. Y.: *War Medicine*, 6: 67-71, 1944.
2. SATO, H., OHMI, F.: *Tohoku Journal of Exper. Med.*, 21: 411-432, 1933.
3. BODO, R. C., CO TUI, F. W., BENAGLIA, A. E.: *THIS JOURNAL*, 61: 48-57, 1937.
4. DEANESLEY, RUTH: *Amer. Journ. of Anat.*, 47: 475-509, 1931.

5. IVY, A. C., GOETZL, F. R., HARRIS, S. C., BURRILL, D. Y.: Quart. Bull. N. U. M. S., 18: 298-306, 1944.
6. EDDY, N. B.: THIS JOURNAL, 33: 43-68, 1928.
7. MOLITOR, H., AND LATVEN, A.: Current Researches in Anesthesia and Analgesia, 16: 127-133, 1937.
8. TURNER, C. D.: Anat. Review, 73: 145-162, 1939.
9. ANDERSON, EVELYN: Essays in Biology, Univ. of Calif. Press, Berkeley and Los Angeles, 33-49, 1943.
10. SNEDECOR, G. W.: *Statistical Methods*, Iowa State College Press, Ames, Iowa, 1946.
11. PUHARICH, V., AND GOETZL, F. R.: Perm. Found. Med. Bull., 5: 19-22, 1947.

COMPARATIVE EFFECTIVENESS OF FIVE ANTIHISTAMINICS VS. HISTAMINE-INDUCED SPASM IN CANINE THIRY-VELLA LOOPS

BRADFORD N. CRAVER, ANNE CAMERON AND FREDRICK F. YONKMAN*,
*Research Department, Division of Pharmacology, Ciba Pharmaceutical Products, Inc., Summit,
New Jersey*

Received for publication February 13, 1948

The history of the investigations whence have emerged our present views of the significance of the rôle of histamine in allergic states has been too recently and thoroughly reviewed to warrant its repetition (1-4). Similarly the history of the logical quest for antihistaminic drugs that the development of this theory stimulated has been reviewed (5-7). Since several antihistaminic drugs are now being clinically employed either here or on the Continent, it seemed worthwhile to try to determine if they acted by the same mechanism. It appeared that indirect evidence for deciding this issue would be forthcoming if a preparation could be found such that in repeated experiments a minimally effective dose (M.E.D.) of a given antihistaminic could be expected to produce the same effect, assuming also that a moderate decrease in that dose would eliminate the effect, since it would then be possible to administer one-half the respective M.E.D.'s of two antihistaminics, or one-third those of three to determine if their effects were strictly additive. To this end nine dogs were surgically prepared with the technique described by Thiry (8), as subsequently modified by Vella (9). They were of both sexes, indeterminate breeds and averaged 19 kg. in weight.

PROCEDURE. The dogs were allowed no food for at least twelve hours before the experiment. The animals were anesthetized with Nembutal administered either intravenously in a dose of 30 mg./kg. or intraperitoneally in a dose of 35 mg./kg. Rarely was it necessary to administer supplementary Nembutal during the experiment. The systemic effects of histamine are too great to make practical the use of unanesthetized animals.

The gut motility was kymographically recorded by means of a balloon inserted into the loop and connected to a bromoform-manometer. A water-manometer was used for some of the earlier experiments but the excursions produced thereby were excessive. The balloon was made in the classical manner by inserting a 14 Fr. rubber catheter into a condom which was tied off about five inches from the inserted end of the catheter. The tie was made over a $\frac{3}{4}$ inch piece of glass tubing of 4 mm. in diameter which had been inserted into the catheter to prevent constriction of the catheter's lumen when the condom was tied in place with cotton thread. Because the single orifice of the catheter could have been occluded by a spasm of the gut, ten or twelve additional openings were made by a hot needle in the portion of the catheter to be included by the condom. Larger openings make the catheter too flexible for ease of insertion into the loop. In the first experiments 20 cc. of air was injected into the closed system but it subsequently seemed more desirable to inject only enough air to provide a differential of about three inches between the fluid levels in the arms of the manometer (equivalent to 21.7 cm. of water).

* The authors wish to express their thanks to Dr. Harry W. Hays who surgically prepared the dogs.

INJECTIONS. All doses of histamine were diluted with 0.9% saline to a total volume of 1.0 ml. and injected rapidly. Such injections were made at intervals of fifteen minutes except when spontaneous contractions would have obscured the response, at which times injections were delayed until the tonus of the gut had decreased to its control level. All doses of antihistaminic drugs were diluted with 0.9% saline to a total volume of 5.0 ml. and injected at the rate of 1.0 ml. per minute, beginning five minutes after the last injection of histamine, since preliminary experiments had indicated that the rapid injection of more concentrated solutions caused hypotension and, in the case of some of the drugs, an interfering stimulation of the gut. Histamine was administered again five minutes after the injection of the antihistaminic had been completed. A burette was arranged for the injections to lessen the incidence of thrombosis. All drugs were washed into the vein with 5 ml. of sterile physiological saline.

Five antihistaminic drugs were studied: 1. N,N-Dimethyl-N'-benzyl-N'-phenylethylenediamine Hydrochloride (Antergan); 2. 2-(N-Phenyl-N-benzylaminomethyl)-imidazoline Hydrochloride (Antistine); 3. β -Dimethylaminoethylbenzhydryl Ether Hydrochloride (Benadryl); 4. N,N-Dimethyl-N'-(p-methoxybenzyl)-N'-(α -pyridyl) ethylenediamine Hydrochloride (Neantergan); 5. N,N-Dimethyl-N'-benzyl-N'-(α -pyridyl)-ethylenediamine Hydrochloride (Pyribenzamine).

The first experiment for each dog involved a two hour recording of the spontaneous motility of his Thiry-Vella loop. The second recorded his response to eight suitable like doses of histamine given at 15 minute intervals since that gave assurance of the duplicability of response. A dose was considered suitable if it caused an elevation of tonus of at least $1\frac{1}{2}$ inches and not more than 4 inches on the kymographic record. Such doses lay in the range of 10 to 20 γ /kg. After completion of the preliminary control experiments, the minimally effective dose (M.E.D.) of each drug was established for each dog. Since all experiments were initiated by two consecutive isometric control responses to the same dose of histamine, after which the test drug was given, the M.E.D. of the antihistaminic was originally defined as the smallest which would eliminate the response to the first dose of histamine given subsequently to the antihistaminic, allow a partial response to the next dose and a response equivalent to the control after the following dose. Inevitable physiological variations made desirable a broadening of this definition to permit the acceptance of any experiment in which a response of like magnitude to the control was elicited by no later dose of histamine than the fifth after the administration of the antihistaminic, presupposing that the intervening responses after the eliminated first response had been partial. Occasionally a loss of responsiveness to the test doses of histamine necessitated doubling or tripling of the test dose before a response comparable to the controls could be elicited. This phenomenon was characteristic of one or two of the test dogs but was not correlated with the test drugs.

RESULTS AND DISCUSSION. The results of this study, summarized in Table 1, are based on over 200 experiments. The estimation of the M.E.D. for each antihistaminic in each dog usually involved several experiments but when once determined for a particular dog was readily duplicable in that animal. The M.E.D. for a given drug in a particular dog was a reproducible value but frequently differed widely from the M.E.D. of the same drug in other dogs. Once the M.E.D. had been established for each drug it became possible to secure indirect evidence for the hypothesis that they act by the same mechanism. (Typical results may be seen in figures 1 through 5.) They were given in various combinations of two drugs employing one-half the M.E.D. of each (figure 6) and in various combinations of three drugs employing one-third the M.E.D. of each (figure 7). Thirty-one experiments were performed using two drugs in combination and ten employing three drugs in combination. All of these

various combinations acted as one would have expected a single drug administered in its M.E.D. to act. Since the actions of these drugs proved to be strictly additive one may assume as probable that they act by the same mechanism.

TABLE 1

DOG	SEX	DOSE OF HISTAMINE $\mu\text{g./kg}$	MINIMALLY EFFECTIVE DOSE ($\mu\text{g./kg}$) ¹				
			Antergan	Antistine	Benadryl	Neoantergan	Pyribenzamine
Brad	M	20	1000	3000+	1000	150	150
Butch ¹	M	10	—	—	—	—	50
Don....	M	10	1000	2000-3000	1000	200	200
Fritzi ²	F	20	—	—	—	—	C.100
Gene	M	20	700-800	3000+	300	40	40
Jessie	F	10	1000	3000	750	25	100
Joe. . .	M	20	1000	3000+	1000	200	200
Nickie. . .	M	20	750	3000	250	50	20
Rusty ³	F	10	—	—	—	—	200

¹ Sacrificed after auto-evulsion of the loop.

² Experiments abandoned because of intestinal stricture.

³ Died after accident.

⁴ The validity of each of the above M.E.D.'s, as defined earlier in the paper, was confirmed by at least one duplication.

THIRY-VELLA LOOP
AUG 22, 1946
DOG-"BRAD"-M-15 42 KG
NEMBUTAL ANES
DOSES PER KG.
ABBREV
H = HISTAMINE PO_4
ATS = ANTISTINE
E = END OF INJECTION

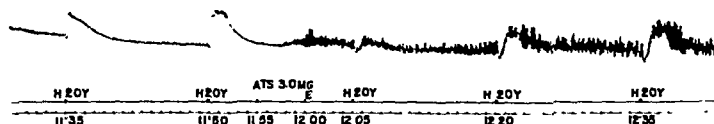


FIG. 1. THE NEGATION OF A STANDARD HISTAMINE-INDUCED SPASM BY M.E.D. OF ANTISTINE IS DEPICTED

Usually after the administration of the antihistaminic the response to histamine was initiated by a brief but marked inhibition of the intestinal motility, a peculiarity rarely seen, and to a lesser degree, in the control responses to histamine. This inhibition so resembled a response to epinephrine that one dog was unilaterally adrenalectomized and a second bilaterally adrenalectomized in an effort to determine if the inhibition were due to epinephrine suddenly liberated from the adrenals. This seemed possible in view of the report by Gibbs and McClanahan (1937) (10) that histamine can cause secretion of saliva from the

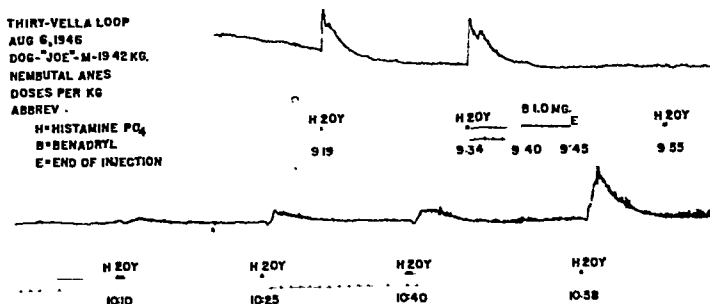


FIG. 2. THE NEGATION OF A STANDARD HISTAMINE-INDUCED SPASM BY M.E.D. OF BENADRYL IS DEPICTED

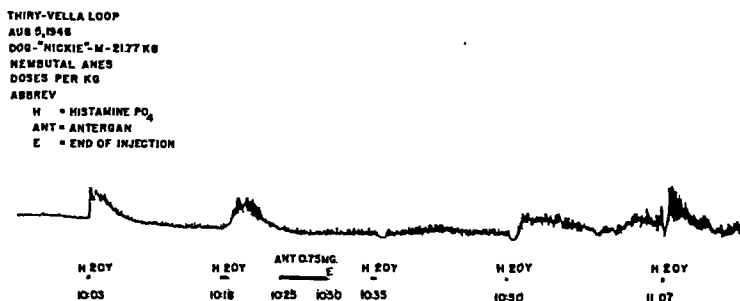


FIG. 3. THE NEGATION OF A STANDARD HISTAMINE-INDUCED SPASM BY M.E.D. OF ANTERGAN IS DEPICTED

THIRY-VELLA LOOP
JUNE 14, 1946
DOG-"GENE"-M-13.96 KG.
NEMBUTAL ANES.
DOSES PER KG
ABBREV. :

H = HISTAMINE PO_4
NA = NEOANTERGAN
E = END OF INJECTION

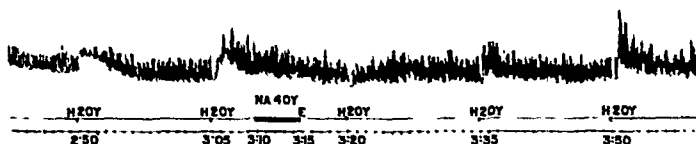


FIG. 4 THE NEGATION OF A STANDARD HISTAMINE-INDUCED SPASM BY M.E.D. OF NEOANTERGAN IS DEPICTED

submaxillaries. It is well known that after the removal of one adrenal the remaining gland hypertrophies. Had the inhibition preceding the response to

histamine been due to epinephrine liberated from the adrenal medulla we had anticipated that it would be absent in the bilaterally adrenalectomized animal and in the unilaterally adrenalectomized dog would be temporarily decreased until the remaining gland had hypertrophied. Since neither anticipated result

THIRY-VELLA LOOP

JUNE 11, 1946

DOG-"DON"-M-20.42 KG.

NEMBUTAL ANES.

DOSES PER KG.

ABBREV. :

H = HISTAMINE PQ_3

PBZ = PYRIBENZAMINE

E = END OF INJECTION

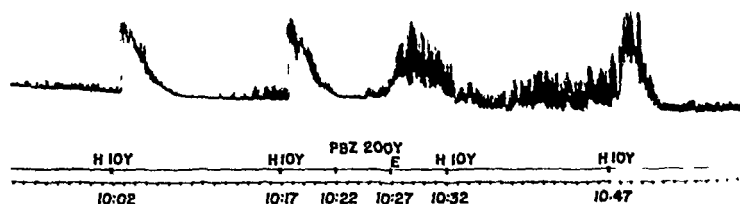


FIG. 5. THE NEGATION OF A STANDARD HISTAMINE-INDUCED SPASM BY M.E.D. OF PYRIBENZAMINE IS DEPICTED

SEPT 20, 1946
"JOE"-19.4 KG - NEMBUTAL
CANINE T.V. LOOP
DOSES PER KG
H=20Y HISTAMINE
B=500Y BENADRYL
PBZ=100Y PYRIBENZAMINE

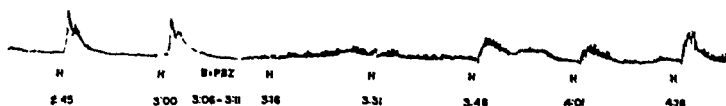


FIG. 6. In this experiment each of the antihistaminics employed was injected in one-half of its M.E.D. and the resultant inhibition of the histamine-induced spasm was such as might have been anticipated after the injection of the M.E.D. of either of the drugs. This suggests, as does Figure 7 also, that the antihistaminics are additive in their action.

was seen and the inhibitory phase remained unaltered, one must conclude it was not due to epinephrine. The contractile responses to histamine in the adrenalectomized dog were prolonged, however, so the normal brevity of the response would appear attributable in part to epinephrine released by the histamine. Only one other possible explanation has occurred to us: that histamine has a

biphasic action and that its inhibitory phase (sympathetic ganglionic stimulation) is more marked after an antihistaminic has depressed preferentially its excitatory phase. This and other peculiarities in the responses to histamine are considered in a following report.

Sherrod and his associates (11) reported that Neoantergan and Pyribenzamine stimulated the duodenum but that Benadryl did not. This may be attributable to the appreciable anti-acetylcholine activity of the last drug. Since the former two drugs exhibit that quality but weakly, this is one respect in which their

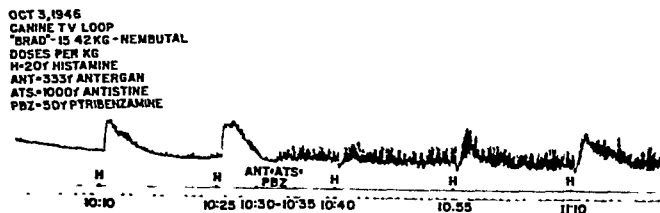


FIG. 7. In this experiment, as may be seen from Table 1, each antihistaminic was injected in a dose representing one-third of its M.E.D. The resultant inhibition of the histamine-induced spasm was such as might have been expected after the injection of a M.E.D. of any one of the drugs. This suggests therefore that the drugs act by an additive mechanism.

pharmacological actions differ and the possible significance of this difference for the present study cannot now be assayed. Hoekstra and Steggerda (12) found that Pyribenzamine invariably increased the amplitude, contraction-frequency, or both in the colon of the dog. It is not surprising therefore that the drugs acted similarly upon the ileum. Except for the stimulating action upon the gut exerted by Neoantergan and Pyribenzamine, and occasionally by Antistine, none of the antihistaminics in the doses employed produced any observable side effects.

SUMMARY

The five antihistaminics: Antergan, Antistine, Benadryl, Neoantergan and Pyribenzamine were effective in non-toxic doses in preventing a histamine-induced spasm of the ileum in dogs with Thiry-Vella Loops. Since the drugs proved additive in their actions, it is presumed that they act by the same or very similar mechanisms.

BIBLIOGRAPHY

- (1) CODE, C. F.: *Ann. Allergy* 2: 457, 1944.
- (2) DRAGSTEDT, C. A.: *Quart. Bull. Northwest. Univ. Med. School* 17: 102, 1943.

- (3) ROCHA E SILVA, M.: J. Allergy 15: 399, 1944.
- (4) Symposium of New York Academy of Sciences (in press).
- (5) FEINBERG, S. M.: J. Allergy 17: 217, 1946.
- (6) FRIEDLAENDER, S.: Am. J. Med. 1: 174, 1946.
- (7) MAYER, R. L.: J. Allergy 17: 153, 1946.
- (8) THIRY, L.: Sitzung berichte d. Akad. Wien. Mathem.—naturw. kl. 1: 50, 77, 1864.
- (9) VELLA, L.: Moleschotts Untersuchungen zur Naturlehre 13: 40, 1882.
- (10) GIBBS, O. S.: AND McCLANAHAN, H. H., THIS JOURNAL, 61: 218, 1937.
- (11) SHERROD, T. R., AND SCHLOEMER, H. F., AND LOEW, E. R.: Fed. Proc. 5: 202, 1946.
- (12) HOEKSTRA, J., AND STEGGERDA, F. R.: Fed. Proc. 5: 48, 1946.

THE INFLUENCE OF DIPHENHYDRAMINE HYDROCHLORIDE AND EPINEPHRINE ON GLUCOSE METABOLISM IN THE RABBIT*

GRAHAM CHEN AND ISABEL G. CLARKE

Research Laboratories, Parke, Davis and Company, Detroit, Michigan

Received for Publication February 17, 1948

Diphenhydramine·HCl and other antihistamine agents have been shown to potentiate the effect of epinephrine on blood pressure (1, 2, 3, 4, 5). It has been speculated that these antihistamine substances may interfere with the elimination of epinephrine in the body, probably by the inhibition of enzymatic processes, which destroy the latter (6, 7).

A study of the influence of diphenhydramine·HCl on blood glucose would, therefore, reflect not only its pharmacological effect on carbohydrate metabolism, but would also reveal the mechanism involved in the joint action of diphenhydramine·HCl and epinephrine on hyperglycemia and probably also on blood pressure. The present investigation was undertaken with these objectives in mind.

EXPERIMENTAL. Blood glucose was determined by the ceric sulfate microtitration method of Miller and Van Slyke (8). Diphenhydramine·HCl and epinephrine were given subcutaneously. One-tenth of a cubic centimeter of blood was drawn from the marginal ear vein of a rabbit at hourly intervals until a maximal glucose level had been reached usually 2 to 4 hours after the injection of diphenhydramine and epinephrine. All animals were fasted for 24 hours prior to experimentation.

In some experiments 30–40 mgm./kg. of pentobarbital sodium given intraperitoneally, was used to antagonize the stimulating effect of diphenhydramine·HCl (9). The rabbits were anesthetized before the administration of diphenhydramine and epinephrine and kept in a depressed state throughout the experiment.

RESULTS AND DISCUSSION. *Epinephrine—Hyperglycemia.* Our first attempt was to work out a quantitative relationship between the dose of epinephrine and hyperglycemia. This information is essential in order to understand the mechanism involved in the combined effect of epinephrine and diphenhydramine on blood glucose.

The curve in figure 1 was made by plotting the maximal increase in blood glucose against the dose of epinephrine, each point on the ordinate being an average value of 5 to 8 determinations obtained in different rabbits. When $\text{Log } (K - Y)/Y$ (Y = maximal increase in blood glucose for each dose of epinephrine, K = the asymptotic value of Y) is plotted against the dose, a straight line is attained. This indicates that the relationship between dose of epinephrine

* Benadryl Hydrochloride and Adrenalin—Parke, Davis & Company's trademarks for Diphenhydramine Hydrochloride and Epinephrine respectively—were used in the investigation.

and hyperglycemia may be expressed by a logistic function: $Y = \frac{K}{1 + ae^{bx}}$
 (10), in the present case, $Y = \frac{260}{1 + 7.1 e^{10x}}$ (X = dose of epinephrine; a , b , K
 = constants determined graphically).

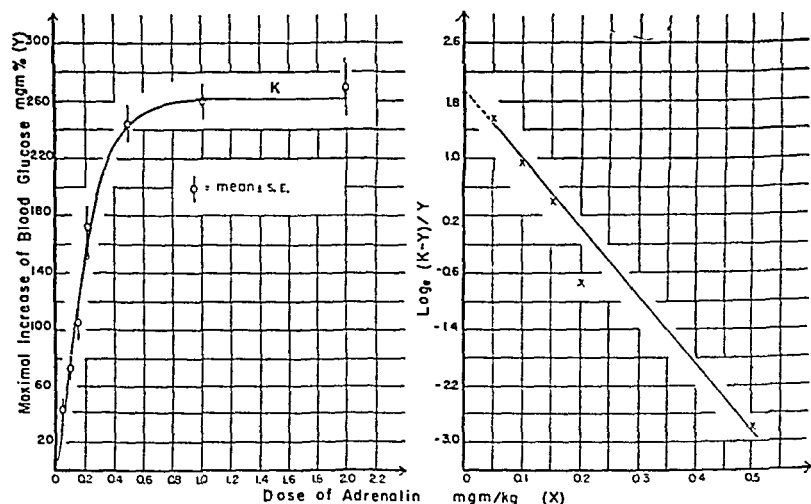


FIG. 1

TABLE 1

Effect of diphenhydramine-HCl on blood glucose

NUMBER OF RABBITS	DOSE (S.C.) mgm./kg.	BLOOD GLUCOSE FASTING VALUE (Av. \pm S.E.)	MAX. VALUE (Av. \pm S.E.) (mgm. %)	INCREASE DIFF. \pm S.E.	P	REMARKS
6	2.5	76.1 \pm 3.2	84.3 \pm 2.6	8.2 \pm 4.5	0.1	
6	5.0	82.0 \pm 2.5	106.3 \pm 4.1	24.3 \pm 4.8	<0.01	
6	10.0	94.7 \pm 2.4	121.3 \pm 4.2	25.7 \pm 4.8	<0.01	
6	25.0	84.9 \pm 1.7	126.0 \pm 13.1	41.1 \pm 13.2	0.01	
6	50.0	89.5 \pm 3.8	149.8 \pm 13.6	60.3 \pm 14.1	<0.01	Three dead 1-2 hours later.
2	100.0	88.0	115.10	27.0		Both dead $\frac{1}{2}$ hour later.

THE EFFECT OF DIPHENHYDRAMINE-HCl ON BLOOD GLUCOSE. The results in Table 1 indicate that diphenhydramine caused a significant increase in blood glucose at 5 mgm./kg. subcutaneously. About 60 mgm. per cent increase of glucose was found at 50 mgm./kg.; half of the rabbits receiving this dose, however, died of convulsive seizures within 1 to 2 hours. In view of the excitement observed in these animals, the hyperglycemic effect of diphenhydramine-HCl was suspected to be the result of its central nervous stimulating action.

In differentiating the central and the peripheral action of diphenhydramine-HCl on blood glucose, experiments were conducted in anesthetized rabbits (11). The data in Table 2 indicate that diphenhydramine did not affect the

TABLE 2
Effect of pentobarbital on diphenhydramine-HCl hyperglycemia

NUMBER OF RABBITS	PENTOBARBITAL I P.	EPINEPHRINE (S C)	DIPHENHYDRAMINE HCl (S C)	BLOOD GLUCOSE		INCREASE DIFF. \pm S E	P
				Fast Val ($\bar{A} \pm S.E.$)	Max Val* ($\bar{A} \pm S.E.$)		
	mgm/kg	mgm/kg	mgm/kg.	(mgm %)			
6	0	0	0	89.6 \pm 3.6	93.3 \pm 4.1	8.7 \pm 6.0	>0.1
6	40	0	0	90.8 \pm 1.4	93.4 \pm 2.4	7.6 \pm 3.0	>0.2
2	40	0	40	93.0	105.5	12.5	
2	40	0	20	84.0	92.0	6.0	
5	0	0.10	0	96.0	172.0	76.0 \pm 5.6	>0.1
5	40	0.10	0	102.0	202.2	100.2 \pm 18.2	

* Maximal value during 4 hourly intervals

TABLE 3
(A)
Pentobarbital and blood glucose in the rabbit

RABBIT NUMBER	BLOOD GLUCOSE (mgm %)					
	1	2	3	4	5	6
Fasting level						
Group I	90	86	91	90	93	95
Group II	99	78	77	90	98	93
Three hours later						
Group I (Without Pentobarbital)	92	93	97	95	107	105
Group II (With Pentobarbital 40 mgm/kg)	108	84	85	102	108	103

(B)
Analysis of Variance

	SUM OF SQUARE	D F	VARIANCE	F	5%
Between groups	416.8	3	138.9	2.1	3.49
Within groups	1315.8	20	65.8	(Insignificant)	
Total	1732.6	23			

blood glucose levels in anesthetized animals, whereas epinephrine produced the usual hyperglycemic effect. The increase of blood glucose following the injection of a large dose of diphenhydramine-HCl was a result of its central action.

Anesthesia in these experiments did not have any effect on glucose levels of the blood; this is shown by an analysis of variance of blood sugar levels of anesthetized and unanesthetized animals. In Table 3 are values of fasting blood sugar

of 2 groups of 6 rabbits each and sugar levels taken 3 hours later with and without anesthesia. This experimental design gives us the variances within groups and between groups in reference to anesthesia and to the time at which the blood

TABLE 4

The combined effect of epinephrine and diphenhydramine-HCl with and without anesthesia

EXPT. NO.	NO. OF RABBITS	PENTOBARBITAL (mgm./kg. I.P.)	EPINEPHRINE (mgm./kg. S.C.)	DIPHENHYDRAMINE (mgm./kg. S.C.)	BLOOD GLUCOSE MAXIMUM INCREASE (mgm. %) (Av. \pm S.E.)	DIFFERENCE BETWEEN EXPTS.	P
1	2	0	0.1	0	35	32.2 \pm 24.3 (3-4)	0.1
	2	0	0	20	51		
	2	0	0.1	20	85		
2	4	0	0.1	0	57.5 \pm 4.3		
	2	0	0	20	25.0		
	4	0	0.1	20	111.8 \pm 11.7		
3	5	40	0.1	0	100.2 \pm 18.2		
4	6	40	0.1	40	68.0 \pm 11.9		

TABLE 5

The effect of epinephrine and cocaine on blood glucose in the rabbit under pentobarbital

EXPT. NO.	NO. OF RABBITS	PENTOBARBITAL (mgm./kg. I.P.)	EPINEPHRINE (mgm./kg. S.C.)	COCAINE HCl (mgm./kg. S.C.)	BLOOD GLUCOSE FASTING (Av.)	MAXIMAL (Av.)	DIFF.
						(mgm. %)	
1	2	0	0	10	80	104	24
2	2	0	0	25	79	113	34
	2	0	0	50	81	126	45
3	2	40	0	25	91	85	-6
4	1	40	0	25	87	79	-8
	2	40	0.1	25	90	153	63
	2	0	0.1	0	94	158	64
5	2	40	0.1	50	80	183	103
	2	40	0.1	0	88	186	98

sample was drawn. The variance between groups as compared with that within groups is statistically not significant (12).

THE COMBINED EFFECT OF EPINEPHRINE AND DIPHENHYDRAMINE-HCl ON BLOOD GLUCOSE. To investigate the joint action of epinephrine and diphenhydramine-HCl, the doses of epinephrine (0.1 to 0.2 mgm./kg.) were so chosen that its hyperglycemic effect, combined with that due to diphenhydramine-HCl,

would be along the steep portion of the epinephrine-response curve (figure 1). The hyperglycemic action of diphenhydramine·HCl was assumed to be similar to that of epinephrine. The data in Table 4, obtained in both anesthetized and unanesthetized rabbits, however, reveal that the joint effect of epinephrine and diphenhydramine on blood glucose is additive, but different in nature. The component due to diphenhydramine·HCl could be inhibited by anesthesia. Apparently, the mechanism involved in the influence of diphenhydramine·HCl upon the effect of epinephrine on blood pressure is different from that on blood glucose.

THE COMBINED EFFECT OF COCAINE AND EPINEPHRINE ON BLOOD GLUCOSE. An atropine-like property of diphenhydramine·HCl has been reported by a number of investigators (13, 14). On the other hand, the potentiating action of diphenhydramine·HCl upon epinephrine on the blood pressure, its local anesthetic action and its mydriatic action superimposing on that of atropine, appear to point to a cocaine-like property. It would be of interest, therefore, to investigate also the influence of pentobarbital anesthesia on the joint effect of cocaine and epinephrine on blood sugar. The results in Table 5, obtained under similar experimental conditions for diphenhydramine and epinephrine, indicate that cocaine likewise produced an increase of blood sugar by central nervous stimulation. It did not potentiate the effect of epinephrine on hyperglycemia.

SUMMARY

The logistic function was found to fit the dose-response curve of epinephrine-hyperglycemia.

The combined effect of diphenhydramine and epinephrine on blood glucose was investigated; their joint action on hyperglycemia was shown to be additive but different in nature. The hyperglycemic effect of diphenhydramine·HCl was apparently due to its central nervous stimulation since it could be inhibited by a depressant.

REFERENCES

1. PARROT, J. L.: *Compt. rend. Soc. de Biol.*, **137**: 378, 1943.
2. BOVET, D., HORCHOIS, R., AND FOURNEI, J.: *Compt. rend. Soc. de Biol.*, **138**: 165, 1944.
3. LOEW, E. R., MACMILLAN, R., AND KAISER, M. E.: *THIS JOURNAL*, **86**: 229, 1946.
4. LEE, H. M., DINWIDDLE, W. G., AND CHEN, K. K.: *THIS JOURNAL*, **90**: 83, 1947.
5. SHERROD, T. R., LOEW, E. R., AND SCHLOEMER, H. F.: *THIS JOURNAL*, **89**: 247, 1947.
6. YONKMAN, F. F., CHES, D., MATHIESON, D., AND HANSEN, N.: *THIS JOURNAL*, **87**: 256, 1946.
7. YONKMAN, F. F.: *Am. J. Dig. Dis.*, **14**: 360, 1947.
8. MILLER, B. F., AND VAN SLYKE, D. D., *J. Biol. Chem.*, **114**: 582, 1936.
9. GRUHIT, O. M. AND FISKEN, R. A., *THIS JOURNAL*, **89**: 227, 1947.
10. REED, L. J., AND BERKSON, J., *J. Phys. Chem.*, **33**: 760, 1929.
11. CAMPBELL, D. AND MORGAN, T. N., *THIS JOURNAL*, **49**: 459, 1933.
12. SNEDECOR, G. W., *Statistical Methods*, **179**: 1940.
13. LOEW, E. R., KAISER, M. E., AND ANDERSON, M. J.: *Fed. Proc.*, **5**: 190, 1946.
14. MCGAVACK, T. H., ELLIS, H. AND BOYD, L. J.: *Am. J. Med. Sc.*, **213**: 418, 1947.

SOME STUDIES ON THE PHARMACOLOGY OF RELAXIN¹

M. X. ZARROW² AND W. L. MONEY³

The Biological Laboratories, Harvard University, Cambridge, Massachusetts

Received for publication February 17, 1948

Active and stable preparations of relaxin have been available for a number of years (1, 2, 3, 4). The early work of Fevold, Hisaw and Meyer (2) indicated that relaxin is probably of a polypeptide nature and can be precipitated with alcohol or as a picrate. This substance was originally obtained from the corpora lutea of sows and has since been extracted from the whole ovary of pregnant and non-pregnant sows (5). However, no investigations have been reported with regard to the pharmacological properties of this material. In view of the recent interest in the physiology of relaxin, and the evidence that it not only causes relaxation of the symphysis pubis of the guinea pig (6, 7, 8) and the mouse (9) but may also play an additional role in the bodily economy (10, 11), it was decided to study certain pharmacological aspects of this substance which might be of value for future experimental and clinical studies, and might also aid in the understanding of the mechanism of its action.

The work reported in this paper is concerned with the rate of disappearance of relaxin from the blood stream and, its speed of action as measured by the effect on the pubic symphysis of the guinea pig. In addition, studies are reported on the possible role of relaxin in the production of antibodies and on the toxicity of several highly purified preparations.

MATERIALS AND METHODS Assays for the relaxin content of blood serum and urine were carried out on castrated, female guinea pigs using a slight modification of the method reported by Abramowitz, Money, Zarrow, Talmage, Kleinholtz and Hisaw (12). The animals were pretreated with 1 μ g of estradiol daily for 3 days and injected with the assay material on the 4th day. One guinea pig unit (G P U) of relaxin has been defined as that amount which will produce relaxation of the symphysis pubis in two-thirds of a group of 12 or more guinea pigs 6 hours after injection of the relaxative substance. The actual loosening of the pubic ligaments was determined by gently moving each half of the pelvis up and down in an alternate manner. All guinea pigs were palpated in this fashion before injection of the test material and only those animals used that showed a tight pelvis.

Prior to injection, the blood serum was diluted with physiological saline so that a minimal volume of 1 ml was used at each assay. The urine was collected under toluene, dialyzed against running tap water for 24 hours and concentrated according to the method of Albert and Money (13).

The relaxin used in these experiments was prepared from the ovaries of sows and stored

¹ Aided in part by a grant from the Donner Foundation to Professor Frederick L. Hisaw.

² Present Address, Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut

In partial fulfillment of the requirements for the degree of Ph D, Harvard University, Cambridge, Mass

³ Present Address: Thyroid Clinic, Massachusetts General Hospital, Boston, Massachusetts.

as a dry powder (12). This material (J-46) was assayed on several hundred guinea pigs and consistently found to possess a potency of 30 G.P.U. per mgm. dry weight. Prior to treatment, the relaxin powder was dissolved in distilled water, dialyzed against running tap water and NaCl added to bring the salt concentration to 0.85%. A second sample of relaxin used in these experiments was a highly purified preparation (F-1) obtained from Dr. E. H. Frieden, Harvard University, and found to contain 250 G.P.U. per mgm.

EXPERIMENTAL PROCEDURE AND RESULTS. A. *Rate of disappearance of relaxin from the blood stream.* Adult male and female rabbits were injected intravenously with a single dose of relaxin which was calculated to produce a concentration of 10-20 G.P.U. per cc. of blood serum (14). Blood was withdrawn from the marginal vein of the opposite ear at intervals of 5 minutes to 6 hours following injection. At 12 to 24 hours after injection, the animals

TABLE I

The relaxin content of the blood serum of rabbits following a single injection

RABBIT NUMBER	SEX	BODY WT.	RELAXIN INJECTED	RELAXIN CONTENT OF SERUM (G.P. UNITS PER ML.)						
				5 min.	1 hr.	2 hr.	3 hr.	6 hr.	12 hr.	24 hr.
A. Intravenous injection of relaxin (J-46)										
		kg.	G.P. units							
73	M	4.54	3,640	10	5				0.4	
75	M	5.45	4,275	10	5	2			0.5	
74	F	4.08	3,600	8-10	5	2		1		0
72	F	4.20	3,600	10	5			1		0
106	M	4.87	8,000	20	10			2		
B. Subcutaneous injection of relaxin (J-46)										
10	F	4.52	3,000	10	5		2	0.75		0
WA	F	4.30	3,690	10	5			1.0		0
WB	F	5.05	3,690	10	4					
84	M	4.68	3,480	10	5			1.0	0.5	
12	M	1.71	1,740	8						0.2
93	M	5.40	4,905	10	5			0.75		0
97	F	2.90	2,800	10	5				0.4	0
80a	F	4.85	8,000	20	10			2	1	
79	F	5.42	9,000	20	10			2	1	

were exsanguinated by cannulation of the carotid artery as large volumes of blood were required to carry out the relaxin assay.

The data obtained from 5 rabbits injected intravenously with relaxin is shown in Table I. It may be seen that doses between 3,600 and 4,275 G.P. units produced a concentration of 8 to 10 G.P. units per ml. of blood, 5 minutes after injection. An approximate doubling of the dose (8,000 G.P. units) resulted in a concentration of 20 G.P. units per ml. of serum 5 minutes after injection. While these values for the initial concentration of relaxin in the blood may appear high it must be remembered that the assay can "distinguish differences in doses of 50 percent but not less" (12).

In order to compare the rate of disappearance of relaxin from the blood stream we have converted the absolute concentrations of relaxin to a percentage by con-

sidering the value obtained at 5 minutes after injection as 100 percent. Thus all values for the concentration of relaxin in the blood subsequent to the 5 minute determination have been referred to this point and the data plotted in figure 1.

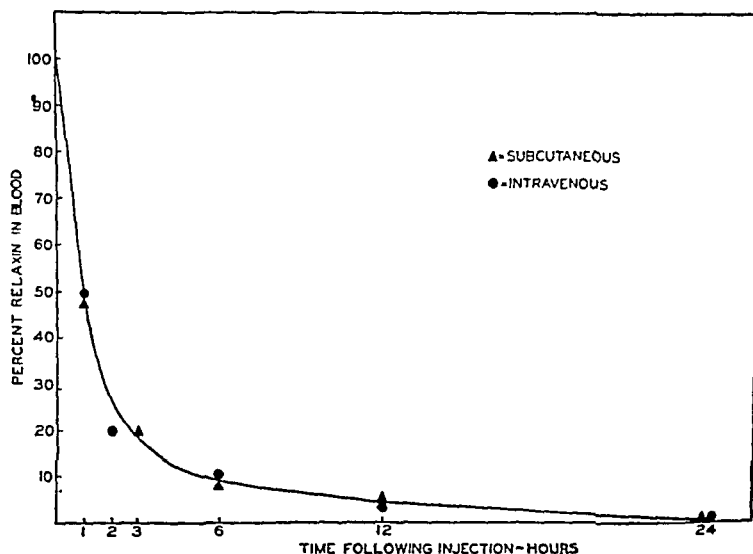


FIG. 1. LOSS OF RELAXIN FROM THE BLOOD OF THE RABBIT AT HOURLY INTERVALS AFTER A SINGLE INJECTION OF THE RELAXATIVE SUBSTANCE

TABLE II

The progressive decrease of relaxin in the blood of rabbits following a single intravenous or subcutaneous injection

TIME AFTER INJECTION	PERCENTAGE OF RELAXIN IN THE BLOOD*	
	Subcutaneous injection	Intravenous injection
5 min.	100	100
1 hr.	48	50
2 hr.	—	20
3 hr.	20	—
6 hr.	9	10
12 hr.	4.5	4
24 hr.	0.5	0

* The percentages are calculated by regarding concentration of relaxin determined, five minutes after the injection, as 100%.

Within 1 hour following injection, a 50 per cent loss of relaxin was observed in 5 rabbits and at 2 hours only 20 per cent remained in the blood stream (Tables I and II). The rate of disappearance then decreased and the curve flattened out so that by 12 hours 96 per cent of the substance had been eliminated from the blood stream.

Assays of urine for relaxin content indicated a concentration of approximately 1 G.P. unit per cc. Thus in a 24 hour period the rabbits excreted only 1 to 4 per cent of the injected relaxin by way of the urine.

Studies similar to those reported above were also carried out following the administration of relaxin by the subcutaneous route (Table I). As may be seen from an examination of the data (Table II and fig. 1) the results were essentially the same as those obtained in the experiment in which the relaxin was injected intravenously. It may also be noted that the maximal concentration of relaxin in the blood following a subcutaneous injection occurred within 5 minutes (Table I).

TABLE III

Time required for relaxation in the guinea pig after subcutaneous, intraperitoneal and intracardiac injections of relaxin (J-46)

HOURS AFTER INJECTION	PER CENT GUINEA PIGS SHOWING PUBIC RELAXATION			
	After subcutaneous injection of 1 G.P. unit (10 animals)	After intraperitoneal Injection of 1 G.P. unit (11 animals)	After intracardiac injection of 1 G.P. unit (15 animals)	After subcutaneous Injection of 100 G.P. units (14 animals)
1	0	0	0	0
2	30	9	0	14
3	30	27	13	35
4	40	36	26	71
5	40	36	40	100
6	70	73	73	100
9	30	45	40	100
12	30	27	26	100
24	0	0	0	36
48	—	—	—	0

B. *Time required for onset of pubic relaxation in the guinea pig.* The rapid appearance of relaxin in the blood following a subcutaneous injection indicated that the time required for a response by the symphysis pubis should not be dependent on the route of administration. The following experiments were designed to test this point. Female guinea pigs weighing 250 grams were castrated and permitted to grow until they attained a weight of 450 to 500 grams. Groups of 10 to 15 animals were treated with 1 μ g of estradiol daily for 3 days followed by relaxin on the 4th day. The first group received 1 G.P. unit of relaxin by the subcutaneous route, the second group received 1 G.P. unit by the intraperitoneal route and the third group received 1 G.P. unit by the intracardiac route. In addition a fourth group received 100 G.P. units of relaxin subcutaneously. The pubic symphyses of the animals were palpated hourly for the first 6 hours and then checked on the 9th, 12th, 24th, and 48th hours for pubic relaxation.

The data show that the maximal percentage of guinea pigs with pubic relaxation was obtained 6 hours after injection of 1 G.P. unit of relaxin (Table III). This took place regardless of whether the hormone was administered by the subcutaneous, intraperitoneal, or intracardiac route. Furthermore it is of interest to note that the curves relating the percentage of guinea pigs showing pubic relaxation to the time after injection are practically identical for all three routes of administration (fig. 2). Pubic relaxation was first apparent in a small percentage

of the animals within 2 hours after injection, the maximal response was obtained in 6 hours and began to disappear by 9 hours. After 24 hours the pubic symphysis of all guinea pigs given 1 G.P. unit of relaxin were no longer relaxed. While the treatment with 100 G.P. units of relaxin did not decrease the time required for the first appearance of pubic relaxation, the time for maximal response was shortened to 4 hours and in all the animals the pubic symphysis remained relaxed for at least 7 hours (fig. 1).

C. Lack of antibody formation. In view of certain characteristics of relaxin and the suggestion that it had a peptide-like structure (2), it was felt desirable to investigate its possible antigenic properties. Consequently an attempt was made to produce antibodies to relaxin.

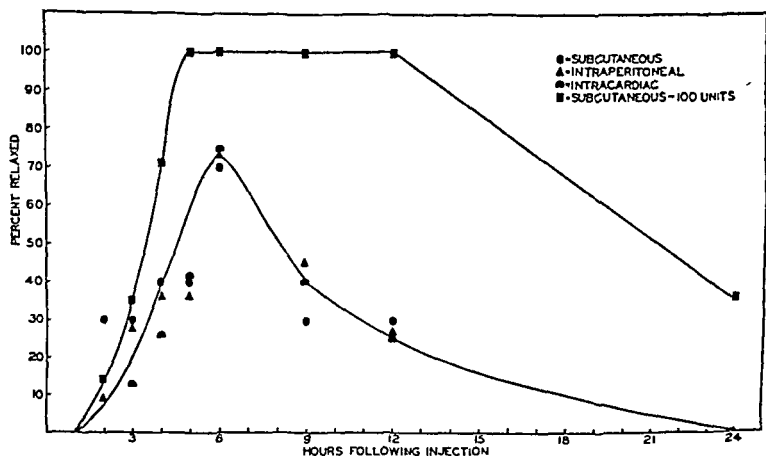


FIG. 2. TIME REQUIRED FOR PUBIC RELAXATION IN CASTRATED GUINEA PIGS PRIMED WITH ESTRADIOL AND GIVEN A SINGLE INJECTION OF 1 G.P. UNIT OF RELAXIN BY THE SUBCUTANEOUS, INTRAPERITONEAL AND INTRACARDIAC ROUTE AND 100 G.P. UNITS OF RELAXIN BY THE SUBCUTANEOUS ROUTE

Rabbit No. 94 received subcutaneous injections of relaxin three times daily on the 1st, 2nd, 25th, 26th and from the 72nd to 78th day. The dose varied from 585 to 1239 G.P. units of relaxin per injection. After a rest period of 19 days the animal was given 250 G.P. units three times daily from the 97th to 131st day. The rabbit was exsanguinated on the 136th day and the serum incubated for 1 hour at 37°C. with relaxin added to make a concentration of 1 G.P. unit per cc. of serum. Assays of this material on the guinea pig showed no loss in activity. Two additional rabbits were injected intravenously with 2,000 G.P. units of relaxin on the 1st and 7th days followed by 2,000 G.P. units intraperitoneally on the 13th day (15). The animals were bled 72 hours after the last injection and the serum incubated with known amounts of relaxin for 1 hour. No loss in potency of the relaxin could be detected.

D. Toxicity of relaxin. The L.D. 50 in mice for a single subcutaneous injection

tion of J-46 was found to be 40,000 G.P. units per kg. body weight (Table IV). When the mice were injected three times daily for 1 day an L.D. 50 of 30,000 G.P. units per kg. body weight was obtained. On the other hand a single intravenous or subcutaneous injection of F-1 at a dose level of 80,000 G.P. units per kg. body weight gave no adverse results (Table IV).

DISCUSSION. These studies indicate that relaxin not only disappears from the blood stream at a fairly rapid rate but that it also enters the circulation with great speed after subcutaneous injection. The maximal recovery of relaxin in the blood 5 minutes after a subcutaneous injection and the similarity in the rate of disappearance after either intravenous or subcutaneous injection would indi-

TABLE IV
Toxicity of relaxin in mice

NUMBER OF MICE	G.P. UNITS OF RELAXIN PER KG. BODY WEIGHT	PER CENT MORTALITY
A. Single subcutaneous injections of relaxin (J-46)		
4	10,000	0
4	20,000	0
12	40,000	50
B. Three subcutaneous injections of relaxin (J-46)		
10	10,000 (total 30,000)	50
C. Single subcutaneous injections of relaxin (F-1)		
4	40,000	0
4	80,000	0
D. Single intravenous injection of relaxin (F-1)		
5	80,000	0

cate that all the relaxative substance has entered the blood within five minutes following injection. A similar rate of disappearance has also been reported for chorionic gonadotropin (16, 17).

This extreme mobility of the relaxative substance is in agreement with the observation that the route of injection has no effect on the time required for relaxation of the symphysis pubis of the guinea pig following injection. It is possible to shorten the latent period for pubic relaxation only by increasing the dosage.

The rapid disappearance of relaxin from the blood stream may be due either to an immediate distribution of this material throughout the body fluids, or to its destruction or elimination in the urine. It has been noted in the present investigation that the maximal amount of relaxin excreted in the urine following injection of 1,740 to 9,000 G.P. units is only 4 per cent. In addition Marder and Money (18) have shown that in the pregnant rabbit the relaxin in the urine never exceeds 5% of the blood level. Thus it would appear that in the rabbit

only a small part of the relaxin is removed by way of the urine and that the body is capable of metabolizing the major portion of the relaxative substance at a fairly rapid rate. Furthermore the rate of production of relaxin must be equal to the rate of destruction and elimination for in both the rabbit (18) and the guinea pig (7) the concentration in the blood is maintained at a constant level for a considerable period during pregnancy. In contrast to the rabbit, equal concentrations of relaxin have been found in the blood and urine of guinea pigs (Zarrow 7, and 19). Nevertheless, during pregnancy the concentration of relaxin in the blood of the guinea pig is only one twentieth that of the rabbit and this may account in part for the difference between the two species.

We were not successful in our attempts to produce antibodies in the rabbit to relaxin. These results, however, were expected for guinea pigs have been given biweekly injections of relaxin for over a year without developing refractoriness to the relaxative substance or showing symptoms of anaphylaxis.

Our results indicate that relaxin has a very low toxicity. Injection of 80,000 G P. units of a highly purified preparation per kg body weight in mice gave no untoward results.

SUMMARY AND CONCLUSIONS

The rate of absorption from the site of injection and disappearance of relaxin from the blood is fairly rapid. Approximately 50% is lost in 1 hour after an intravenous injection and is almost entirely absent by 24 hours. The maximal concentration of relaxin is found in the blood 5 minutes after a subcutaneous injection, following which the rate of disappearance is identical to that observed for an intravenous injection. The latent period between administration of the substance and relaxation of the symphysis pubis of the guinea pig is the same following the injection of 1 G P unit of relaxin by either the subcutaneous, intraperitoneal, or intracardiac routes.

Relaxin is not antigenic and highly purified preparations of the relaxative substance are non-toxic in extremely high doses.

BIBLIOGRAPHY

1. FEVOLD, H. L., HISAW, F. L., AND MEYER, R. K. *Proc Soc Exper Biol and Med*, **27**: 604, 1930.
2. FEVOLD, H. L., HISAW, F. L., AND MEYER, R. K. *Am Chem Soc*, **52**: 3340, 1930.
3. FEVOLD, H. L., HISAW, F. L., AND MEYER, R. K. *Proc Soc Exper Biol and Med*, **28**: 606, 1930.
4. FEVOLD, H. L., HISAW, F. L., AND LEONARD, S. L. *Am Chem Soc*, **54**: 254, 1932.
5. ALBERT, A., MONEY, W. L., AND ZARROW, M. X. *Endocrinol*, **40**: 370, 1947.
6. HISAW, F. L., ZARROW, M. X., MONEY, W. L., TAYLOR, R. V., AND ABRAMOWITZ, A. A. *Endocrinol*, **34**: 122, 1944.
7. ZARROW, M. X. *Proc Soc Exper Biol and Med*, **66**: 488, 1947.
8. TAYLOR, R. V. *Anat Rec*, **99**: 91, 1947.
9. HALL, K. J. *J of Endocrinol*, **5**: 174, 1947.
10. HAMOISKY, M., AND SPARROW, R. C. *Proc Soc Exper Biol and Med*, **60**: 8, 1945.
11. DEWAR, A. D., HALL, K., AND NEWTON, W. H. *J of Physiol*, **105**: 37p, 1946.

12. ABRAMOWITZ, A. A., MONEY, W. L., ZARROW, M. X., TALMAGE, R. V. N., KLEINHOLZ, L. H., AND HISAW, F. L.: *Endocrinol.*, **34**: 103, 1944.
13. ALBERT, A., AND MONEY, W. L.: *Endocrinol.*, **38**: 56, 1946.
14. WENT, S. AND DRINKER, C. L.: *Am. J. Physiol.*, **88**: 468, 1929.
15. BOYD, W. C.: Interscience Publishers: New York, 1943.
16. ZONDEK, B. AND SULMAN, F.: *Vitamins and Hormones*, **3**: 297, 1945.
17. LIPSCHUETZ, A. AND VIVALDI, T.: *C.R. de la Soc. de biol.*, **116**: 87, 1943.
18. MARDER, S. N., AND MONEY, W. L.: *Endocrinol.*, **34**: 115, 1944.
19. ZARROW, M. X.: *Endocrinol.* (in press).

SUGAR ALCOHOLS

XXVI. PHARMACODYNAMIC STUDIES OF POLYOXYALKYLENE DERIVATIVES OF HEXITOL ANHYDRIDE PARTIAL FATTY ACID ESTERS

JOHN C. KRANTZ, JR., C. JELLEFF CARR, JOSEPH G. BIRD AND SALLY COOK

Department of Pharmacology, School of Medicine, University of Maryland, Baltimore

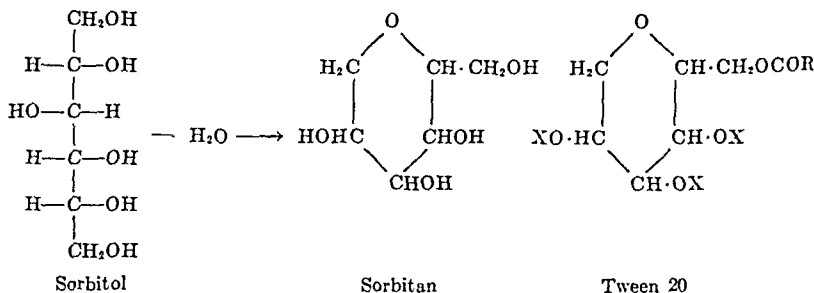
Received for publication February 19, 1948

The mixing of immiscible liquids is a problem in many industries and in medicine. In recent years great strides have been made in the development of new dispersing agents which have simplified many of these difficulties. A series of polyoxyalkylene derivatives of the hexitols, mannitol and sorbitol, and their anhydrides, partially esterified, are being used for this purpose.¹

After toxicological studies in this laboratory had been made, products of this type were made available as emulsifiers and solubilizers in many foods and oil-soluble medicinal products, such as the steroids, vitamins A and D, and essential oils. After years of extensive ingestion of these substances in small quantities in food, no untoward effects have come to our attention.

The availability of these substances by vein, however, is a problem of special pharmacologic and therapeutic interest. If these dispersing agents are available by vein in man, it is possible that these substances might be employed in intravenous fat feeding, treatment of tuberculosis (1), increasing phagocytic activ-

CHEMICAL AND PHYSICAL CHARACTERISTICS. The derivation of the Tweens from the sugar alcohols may be seen in the following formulas:



R = Lauryl Radical, X = Polyoxyethylene Groups (total approximately 20)

Tweens are numbered 20, 40, 60, 80, etc. depending upon the acid esterified with the hexitan. Tween 20 is the polyoxyethylene derivative of sorbitan monolaurate. As a class they are nearly neutral substances; their 5 per cent aqueous solutions or suspensions have a pH range from 5 to 7. Their solubility varies from completely water-soluble to completely oil-soluble. They are non-volatile, stable substances ranging from limpid liquids through viscous oils to waxes. They are non-electrolytes.

¹ The substances used in these experiments were supplied by the Atlas Powder Company in Wilmington, Delaware, and are available under the trademark name "Tween".

ity of white cells (2), making available steroids and fat-soluble vitamins by intravenous therapy. In addition, they might prove valuable in increasing the penetration by chemotherapeutic agents into lipid barriers, e. g. bacterial and tumor cell membranes.

The present communication deals with early toxicity studies in animals, of the polyoxyethylene derivative of sorbitan monolaurate (Tween 20), an unusual allergic type of response in one species (dog), and studies in man to determine the availability of the Tween for intravenous therapy.

TOXICOLOGY. Extensive feeding studies in this laboratory have shown Tween 20 to be innocuous in concentrations of 0.5 to 2 per cent in the diet of the white rat for nearly the life span. Feeding of 1 gm. per day to the *Macacus rhesus* monkey (4 animals, 17 months) produced no significant histologic visceral changes.

TABLE 1
Hemolysis studies with Tween 20

PERCENTAGE TWEEN 20	NO. EXP.	TIME FOR 90 PER CENT HEMOLYSIS
		<i>minutes</i>
10	3	3-4
5	3	10-11
2.5	3	19-20
1.0	6	25-30
0.1	10	90
0.01	10	No hemolysis in 48 hours
0.001	10	No hemolysis in 48 hours

Acute toxicity (white rat intravenous). Fifteen animals were injected with varying quantities of a 10 per cent aqueous solution of Tween 20. In rats weighing approximately 200 grams, 0.5 to 1 cc. produced instant apnea, depression, exophthalmus and narcosis. Subsequent prompt recovery occurred within 2 to 3 minutes. None died over an observation period of 3 days.

Acute toxicity (rabbit intravenous). Nine 2 to 2.5 kg. rabbits were injected intravenously with varying quantities of 5 and 10 per cent solutions of Tween 20. One animal survived an injection of 5 cc. of a 5 per cent solution and 3 animals survived injections of 10 cc. of the same solution. The symptoms were similar to those described in the rat. Two rabbits succumbed to injections of 7.5 cc. of a 20 per cent solution of Tween 20, and 3 died from 5 cc. of a 20 per cent solution of Tween 20. Solutions stronger than 10 per cent do not appear to be well tolerated. Death was almost instantaneous—it was preceded by nystagmus, exophthalmus, gasping and muscular rigidity.

Hemolysis studies. Serum from dogs after an intravenous injection of 1 cc. per kg. of 5 per cent solution of Tween 20 showed evidence of hemolyzed red cells. Accordingly hemolysis studies *in vitro* were conducted.

The freshly shed, defibrinated blood of the dog was used. Solutions of Tween 20 in various concentrations in normal salt solution were prepared. To 10 cc. volumes of these solutions 0.1 cc. blood were added and mixed at $26 \pm 0.5^\circ \text{C}$. The results are shown in table 1.

Tween 20 appears to be a true hemolytic agent, as its capacity to hemolyze red cells is not prevented by isotonic salt solution. The effective hemolytic concentration *in vitro* appears to be above 100 mg. per 100 cc. Previous studies in this laboratory (3) showed that neither the sugar alcohols nor their anhydrides are true hemolytic agents. We have found other Tweens to exhibit less hemolytic activity.

Intravenous injections of 5 cc. of 5 per cent Tween 20 in the dog on 3 consecutive days failed to produce hemoglobinuria.

Chronic toxicity (monkey—intramuscular, subcutaneous and intravenous). Four large *Macacus rhesus* monkeys were selected for the study. Body weight, red cell count, hemoglobin percentage and electrocardiogram Lead II were determined on the normal animals. Two animals were injected with 5 cc. of a 5 per cent solution of Tween 20 intramuscularly and subcutaneously respectively for 20 days. Two animals received the same dosage intravenously. At the conclusion of the experiment the foregoing tests were again carried out; the animals were sacrificed and skeletal muscle, kidney, skin, liver and sternal marrow were studied histologically.

The red cell count, red bone marrow, cardiac rhythm, liver and kidneys, were not significantly affected by the injections. The sites of the intramuscular and subcutaneous injections were found to show definite injury. There was edema under the corium, necrosis of collagen fibers in the fat, capillary engorgement, some residual lymphocytic infiltration and a mild fibroblastic response.

Blood pressure studies (dogs, monkey, cat, rabbit, rat, chicken, guinea pig—intravenous). Intravenous injections of from 1 to 5 cc. solutions of Tween 20 (5 to 50 per cent) produced a short depressor response in the monkey, cat, rat, chicken, guinea pig and rabbit, anesthetized with ether or pentobarbital sodium. Similar injections in the dog anesthetized or unanesthetized produced the same transient fall in blood pressure followed by a slow persistent lowering of the arterial pressure to one-half or even one-fifth its original value. The pressure remained at such a level for from one to three hours, and slowly returned to normal. The degree and duration of this response was greater in the unanesthetized animal. Approximately 100 dogs have shown this phenomenon over a five year period of study.

Blood pressure tracings of the dog and rabbit are shown respectively in figures 1 and 2.

Blood pressure studies (dog). The dog exhibited a reaction to intravenous injections of Tween 20 not elicited in any other species studied. The severity of this reaction varied among dogs. It suggested an allergic response in this species; allergic responses are known to occur in dogs (4). Within one minute after injection of 0.1 cc./kg. of a 5 per cent solution most dogs exhibited signs of weakness, and postural difficulties. Within the next one or two minutes they scratched themselves vigorously, and showed generalized erythema of the skin and mucous membranes. Salivation, nausea and vomiting frequently occurred in the first five minutes. These latter symptoms were very mild in some dogs. All animals recovered in one to two hours.

A dog with a femoral blood pressure of 120 mm. Hg, under local anesthesia was given 1.0 cc. of 5 per cent solution of Tween 20. The blood pressure fell

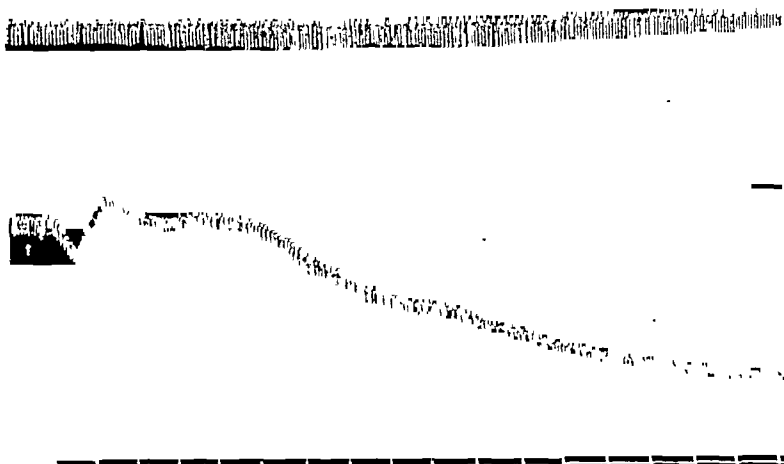


FIG. 1. BLOOD PRESSURE AND RESPIRATION OF DOG UPON INTRAVENOUS INJECTION OF TWEEN 20, 5 PER CENT SOLUTION 1 CC./KG.

Wt. 5.5 kg. Ether anesthesia. Original B.P. 140 mm. Hg. Minimum B.P. 3 min. after injection—50 mm. Hg. Time interval 10 sec.

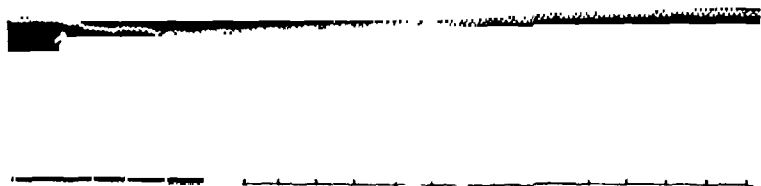


FIG. 2. BLOOD PRESSURE OF RABBIT UPON INTRAVENOUS INJECTION OF TWEEN 20, 5 PER CENT SOLUTION 0.5 CC./KG.

Wt. 2.2 kg. Ether anesthesia. Original B.P. 90 mm. Hg. Full depressor effect 160 mm. Time interval 10 sec.

to 22 mm., the dog remained alert on the table, and exhibited the usual skin flushing and scratching. An hour later this animal was given an additional 160 cc. of the solution which did not produce any further fall in blood pressure

When the experiment was terminated the blood pressure was still below 50 mm., but the animal was able to walk. The animal developed marked hemoglobinuria. Eighteen hours later it was alert and under ether anesthesia the mean carotid blood pressure was 160 mm. Five cc. of Tween 20, 5 per cent, had no effect on the blood pressure, but acetylcholine, epinephrine and tetraethylammonium chloride produced their usual effects. The brain of this animal was then studied with a fat stain, owing to the capacity of Tween 20 to solubilize fat. It was found to exhibit no abnormality.

The depressor action of Tween 20 in the dog is not obliterated by atropine, nicotine, Etamon, or by decerebration.

Blood pressure response of analogous substances. The depressor response is not produced by sorbitol, sorbitan, ethyl laurate, or the hydrolyzed polyoxyethylene chain. However, solutions of crystalline sorbitan monolaurate in 20 per cent alcohol (to effect solution) produced a depressor response but of a lesser degree than that of Tween 20. Commercial sorbitan monolaurate does not produce such response. The mannitan analogue of Tween 20 has the same effect as Tween 20. Tweens 40, 60 and 80 produce the same characteristic depressor effect in the dog.

Other effects of Tween 20. As little as 0.02 cc./kg. of 5 per cent Tween 20 may lower the blood pressure 20 mm. of mercury. Five-tenths cc./kg. always produces the marked fall. Injections of Tween 20 solution produce prompt but transient cardiac acceleration. Only slight changes in pulse rate are observed when the animal is anesthetized.

Electrocardiographic studies in 2 dogs showed a decrease in amplitude of the R spike and T wave in Lead II during the period of hypotension produced by Tween 20.

The respiratory rate with or without anesthesia is not noticeably affected by the foregoing Tween 20 injections.

The blood pressure of the dog responds in the usual manner to epinephrine and to acetylcholine, even when the full Tween 20 depressor response is in effect.

Tween allergy in the dog. A very interesting feature of the Tween action is the absolute refractoriness of the animal to a second injection during and after recovery from a first dose adequate to give the maximum depressor response. Dogs will react with skin flushing, scratching and occasional vomiting upon daily intravenous injection of 0.1 cc./kg. of a 5 per cent solution of Tween 20. They do not develop tolerance. Apparently 0.1 cc./kg. can be metabolized or excreted in less than 24 hours, allowing the animals' sensitivity to redevelop partially toward the end of that period.

Intradermal testing of 10 dogs with 0.01 cc. of 1:200 dilution of Tween 20 in 0.9 per cent sodium chloride solution elicited in each case a pink wheal on the abdomen at least 1 cm. in diameter in 20 minutes. Saline controls were negative. Positive reactions are also seen with a 1:2000 dilution. No reactions are obtained in rats or guinea pigs by these injections. During the refractory period after recovery from a Tween 20 injection no skin response can be elicited in the dog, but a mild reaction may be obtained the next day.

Seven dogs under ether anesthesia were injected with suitable quantities of Benadryl and Pyribenzamine respectively. The response of the dog's blood pressure to an injection of histamine was partially blocked by the previous injections of either of the two antihistaminics. Similarly, the fall in blood pressure elicited by 5 cc. of a 5 per cent solution of Tween 20 was partially obliterated and in 2 cases completely nullified by the antihistaminics.

The demonstration of histamine or a similar substance in the blood of seven Tween-injected dogs and its absence in 3 Tween-injected rabbits (a species showing no prolonged depressor response) was undertaken. From dogs under ether anesthesia a sample of blood was withdrawn. The cells were removed by centrifuging. One cubic centimeter of the clear serum was added to the bath of a smooth muscle preparation of a guinea pig's jejunum. Either no response was elicited or a small contraction of the smooth muscle was observed. After the injection of 5 cc. of a 5 per cent solution of Tween 20 intravenously, when the full depressor response was in effect, another sample of blood was drawn. Serum from this blood produced always a contraction of the guinea pig's jejunum, com-

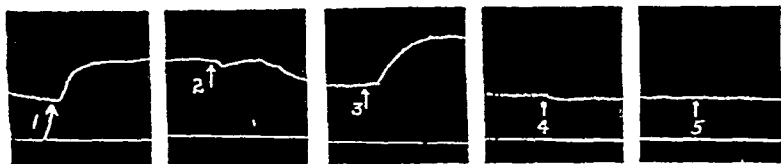


FIG. 3. ISOLATED GUINEA PIG'S JEJUNUM

1. Histamine phosphate, 1 gamma. 2. Serum from etherized normal dog, 1 cc. 3. Serum from etherized dog taken 8 min. after injection intravenously of 5 cc. Tween 20 (0.5 cc./kg.). 4. Pyribenzamine, 1 gamma. 5. Serum from same dog (repetition of #3 above). Volume of bath 30 cc.

parable to the response from 1 gamma of histamine. The response always occurred with the Tween serum and was greater always than that of the control serum, when the latter had evoked an effect.

The effect of the Tween 20 serum on the smooth muscle of the guinea pig was abolished by 0.1 to 10 gamma of Pyribenzamine. Similarly, Pyribenzamine abolished the effect produced by histamine. When responses were elicited by control sera they were not obliterated by Pyribenzamine.

Since serum from a Tween 20-injected dog contains a small amount of Tween 20, calculated not to exceed 30 mg. per cent in our experiment, 1 cc. of this concentration of Tween 20 was added to the smooth muscle preparation and no response was elicited.

Typical tracings of the responses described are shown in figure 3.

Serum from normal and Tween 20-treated rabbits gave identical effects on the jejunum preparation; neither was obliterated by Pyribenzamine.

One dog under ether anesthesia received an infusion of sodium arsenate by vein. Approximately 50 cc. of a 5 per cent solution was administered to a 6 kg. dog. The blood pressure fell from 116 mm. to 64 mm. Hg, presumably through capillary poisoning. The response of the dog's blood pressure to 10

gamma of histamine was markedly reduced. Upon the injection of 5 cc. of 5 per cent Tween 20 solution the characteristic prolonged deep fall in blood pressure was somewhat mitigated. After the blood pressure had fallen upon the injection of Tween 20, histamine produced no further depressor response. However, acetylcholine 1 cc. (1:100,000) further reduced the blood pressure, indicating that the preceding depressor responses were concerned primarily with capillary dilatation.

Upon the assumption that the effect of Tween 20 upon the blood pressure of the dog was due to capillary dilatation owing to histamine release the following experiment was conducted. A 7 kg. dog under ether anesthesia was injected with Tween 20 (5 cc. of a 5 per cent solution). The blood pressure fell to 34 per cent of the normal value. One liter of 6 per cent acacia in normal salt solution was infused into the femoral vein during a 15 minute period. The blood pressure was doubled by this increase in blood volume and this new pressure was maintained over an observation period of 30 minutes.

As the dog manifests a hypersusceptibility to Tween 20 solutions, as shown by the prolonged depressor response, we studied the effect of repeated intravenous injections. One dog received 1 cc. of a 5 per cent Tween 20 solution daily for 3 weeks. At the end of this period the animal was sacrificed. No significant histologic findings were observed in the kidneys, liver or spleen.

Depressor activity in species other than the dog. The fleeting depressor response seen in all animals tested with Tween 20 intravenously begins immediately after injection and lasts from 10 to 60 seconds. The fall in blood pressure ranges from 5 to 30 per cent among various species, and obtains with and without anesthesia. The response varies with the dose, small amounts failing to produce an effect in many animals. A brief compensatory rise in blood pressure usually follows the fall.

In high dilutions (1:10,000) in the Trendelenberg experiment, Tween 20 produces constriction of the leg vessels of the frog.

*Availability in man.*² Intradermal tests similar to those conducted on the dog, guinea pig and rat were carried out on 6 individuals. The intradermal injections were begun with 0.5 per cent Tween 20 solution and the concentrations increased until 5 per cent solutions were used. No wheal greater than that produced by control injections of normal salt solution was produced in man. We therefore concluded that the drug allergy exhibited by the dog to Tween 20 did not obtain in man.

An individual with an extensive carcinomatosis was given 0.5 to 2 cc. of a 5 per cent solution of Tween 20 intravenously. No change in blood pressure was observed. Two other individuals with far advanced pulmonary tuberculosis were given repeated doses, 2.5, 5 and 7.5 cc. of the Tween 20 solution by vein. No changes in blood pressure or pulse occurred and the patients reported no subjective symptoms.

Another individual with congestive heart failure and carcinomatosis was given

² We are indebted to Dr. Lawrence M. Serra and Dr. Lester A. Wall, Jr., for their assistance in these studies in man.

successively 2, 3, 5, 6 and 8 cc. intravenous injections of Tween 20. There were no significant changes in blood count and red cell fragility during the course of the injections over a period of 4 days. There were no blood pressure changes or blood cholesterol alterations.

SUMMARY

1. The parenteral administration of Tween 20 to several species of animals has been investigated. Injections of five per cent solutions subcutaneously and intramuscularly are well tolerated by the monkey. Intravenous injections produce transient depressor responses in all species of laboratory animals tested (except the dog) when given in small doses. Larger doses produce coma and death.

Tween 20 is hemolytic to red cells in concentrations of 100 mg. per cent within 90 minutes *in vitro*.

2. Tweens 20, 40, 60 and 80 produce in the dog a deep and protracted fall in blood pressure.

3. The depressor response appears to be due to the release of histamine or histamine-like substances which act upon the capillary bed.

4. This allergic depressor response is not elicited in the rat, guinea pig, cat, rabbit, *Macacus rhesus* monkey, chicken or man.

5. To 4 hospitalized patients 2 to 8 cc. of Tween 20, 5 per cent solution, has been given intravenously. There were no discernible deleterious effects.

We are of the opinion that intravenous therapy with Tween 20 may be explored cautiously in man. If subsequent studies do not reveal untoward effects not observed in these exploratory injections, many avenues of usefulness may manifest themselves for these substances.

REFERENCES

1. DUBOS, R. J., AND DAVIS, B. D.: J. of Exper. Med., 83: 409, 1946.
2. BERRY, J. L.: Personal communication of Atlas Powder Co.
3. KUNKLE, A., CARR, C. J., AND KRANTZ, JOHN C., JR: Proc. Soc. Exper. Biol. & Med., 42: 438, 1939.
4. WITLIER, F. W.: J. of Allergy, 12: 247, 1941.

QUANTITATIVE MEASUREMENT OF UTERINE RESPONSES USING THE STRAIN GAGE DYNAMOMETER, WITH NOTES ON THE EFFECT OF ANTI-HISTAMINIC DRUGS ON THE RABBIT MYOMETRIUM

S. R. M. REYNOLDS AND IRWIN H. KAISER¹

Department of Embryology, Carnegie Institution of Washington, Baltimore 5, Md.

Received for publication February 28, 1948

The myometrium of experimental animals is commonly employed to test oxytocic, spasmolytic or other musculotropic agents. In general, the uterus is used in one of two ways. Either the uterus is excised and immersed in oxygenated physiological salt solution, or it is used *in vivo* in unanesthetized or anesthetized animals after suitable operative and technical preparation. Whichever method is used, the effect of a drug, or a change in the physiological environment, is judged by simple inspection of the kymographic tracing of myometrial activity. The magnitude of the effect is estimated by noting the qualitative changes in tonus and frequency of rhythmic contractions. When the results are expressed quantitatively, it is usual to employ a series of + or - signs according to the judgment of the investigator or the reader.

Clearly, the most useful method will be one which yields results calculable in terms of absolute force or percentage changes from a normal or control period of activity. In this paper, such a method is described.

The key to the method is the use of the Statham strain gage dynamometer.² This instrument produces linear deflections of the recording element with equal increments of pressure which cause electrical resistance changes in the active elements of the strain gage. These changes are measurable as grams of force (1). Moreover, analysis of the records thus obtained permits distinction between the work done as a result of tone, and work done as a result of rhythmic contractions. Results may be expressed as relative percentage changes or they may be expressed in absolute terms as dynes per minute per gram of myometrium.

METHODS AND PROCEDURES. *Animal preparation.* The preparation of the rabbits is carried out in the following way. Prolonged light anesthesia is induced by intravenous injection of 0.4 cubic centimeters of Dial-Urethane per kilogram of body weight. This is supplemented by ether during operative procedures.

The uterus is exposed by a midline abdominal incision. The intestines and cecum are packed out of the way with gauze and a loop of uterus exposed. In some experiments, two loops of uterus are employed simultaneously. In others, records are made simultaneously from a segment of non-distended uterus in a pregnant rabbit having one uterus rendered sterile by prior ligation of the Fallopian tube, and from within the amniotic sac of a conceptus site in the other uterus. Blunted 18 gauge needles are used. The needle is connected to a Statham dynamometer in the manner described below. The needle is inserted into the caudal end of the loop of uterus isolated as follows. Silk ligatures are passed

¹ Present address: Sinai Hospital, Baltimore 5, Maryland.

² Statham Laboratories, 8222 Beverly Blvd., Los Angeles 36, Calif.

through the broad ligament at two points about one and-a half centimeters apart. The caudal ligature is secured around the three way stopcock as shown in figure 2. Another ligature is then passed around the uterus and needle at the caudal end. This is shown in figures 1 and 2 which demonstrate the unit as a whole and a close up of the insertion of one needle attached to a strain gage into a loop of non distended uterus and another into the amniotic sac of a conceptus site. It will be observed that the needle is placed so that it lies along the long axis of the uterine cornu in order to maintain free communication between the fluid in the uterine cavity and the fluid in the strain gage. Interference with the blood and nerve supply to the uterus is held to a minimum. In the case of a conceptus

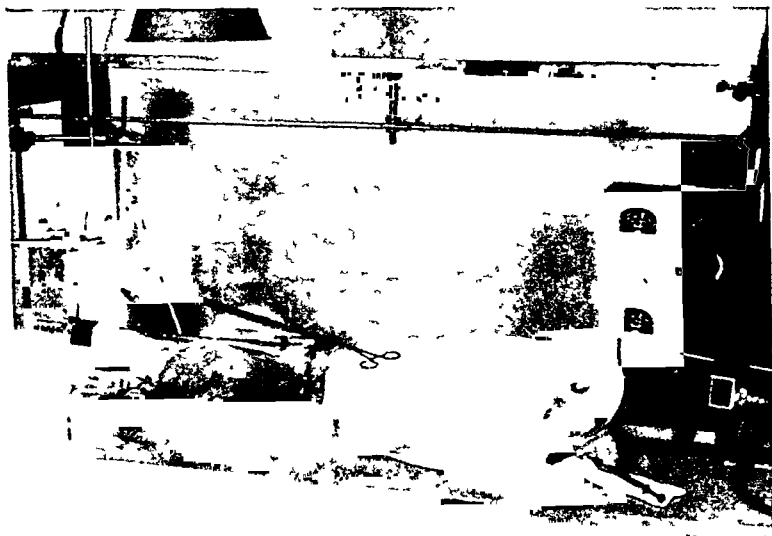


FIG 1 PICTURE OF THREE STRAIN GAGE DYNAMOMETERS EMPLOYED IN RECORDING UTERINE ACTIVITY AND RESPIRATION

Two gages at left are connected to the uterus, one to a distended conceptus site (amniotic sac) and the other to a non distended portion of the contralateral uterus. The gage at the right is connected to an intrapleural trocar and to a water manometer, so that the condition of the rabbit may be noted at all times. Gages are held firmly in just the right position by ball and socket clamps. The black boxes at the rear right are the power supply and control units for two of the strain gages.

site, no ligatures are used. During an experiment, the incision is closed with clamps in order to protect the viscera from the air.

Strain gage dynamometer The Statham strain gage is made ready for use in the following way. The valve head is connected to a pressure bottle containing saline and, by a side arm, to a bottle containing dry ice. For half an hour, carbon dioxide is passed through all tubing in the system and through the valve head in order to displace air. Immediately upon stopping the flow of gas, saline is pumped through the valve until no bubbles pass out with the first flowing saline at the end of the needle. This is attached by the three way adapter shown in figure 2 to a short length of stainless steel flexible metal hose on the strain gage. Residual carbon dioxide is absorbed by the saline. Thus a system free of trapped gas is obtained.

The gage is mounted on rods held in position in locked ball and socket joints close to the level of the loop of uterus into which the needle is to be inserted (see figure 1). By opening

the side-arm of the three-way stopcock from the gage to the atmosphere after the needle, gage, and uterus are in place, the system comes to atmospheric pressure. A record of this true zero level is obtained on the photographic tracing. Upon turning the cock so the uterus connects directly with the gage, the system comes into pressure equilibrium with the uterus. There is virtually no exchange of fluid between the gage and the uterus because the maximum movement of the bellows of the gage is ± 0.0015 inch, and the diameter of the bellows is about 0.25 inch.

The characteristics of the Statham strain gage lend themselves admirably to this type of work. The input current is derived from a six volt source, controlled to give between thirty to fifty milliamperes of current. The current goes directly to the input leads of the gage. The current output, which may be as much as 37 millivolts, is proportional to the unbalance



FIG. 2 CLOSE-UP OF THE INSERTION OF THE NEEDLES CONNECTING THE STRAIN GAGES TO THE AMNIOTIC SAC OF A GRAVID PORTION OF THE UTERUS AND TO THE CORNUAL END OF THE NON-PREGNANT UTERINE HORN

See text for details concerning the method by which the system is rendered air-free and the intrauterine pressures and other observations are obtained. During an experiment the incision is closed.

of the Wheatstone bridge elements of the gage as these are subjected to alteration in tension by pressure or suction on the bellows of the gage. The output current goes directly to the oscillographic recording element. We employ a six channel Hathaway magnetic oscillograph. The elements of this can be varied according to the need for different frequencies and sensitivities in various types of physiological recording. For uterine work, we usually employ elements which have a natural frequency of twenty-five cycles per second, and have a sensitivity of 5600-9600 millimeters per milliamper at one meter distance.

The strain gage dynamometer may be calibrated in either of two ways. It may be connected by a side-arm to a water manometer and deflections recorded as known pressures are applied or it may be calibrated electrically by shunting one input and one output lead of the gage with a suitable electrical resistance. When this is properly adjusted, a deflection equal to fifty per cent of the maximum gage output may be obtained. It should be noted, how-

ever, that by controlling the input voltage and amperage in every experiment, a straight line calibration is obtained which holds true indefinitely.

For recording uterine activity, a gage with a range of ± 1 pound per square inch is used. This, with the current input employed and with the oscillographic recording elements we have used in recordings of uterine activity, yields a deflection on the photographic paper in which 1 centimeter is equal to 55 millimeters of water pressure in the most sensitive arrangement. In the least sensitive arrangement it is equal to 160 millimeters of water. The arrangement employed depends upon the magnitude of physiological activity to be recorded.



FIG. 3. EFFECT OF PITOCIN ON THE UTERUS OF A NON-PREGNANT RABBIT

See text for details of the analysis of this record for work per minute (dynes per gram of myometrium per minute) in terms of tone, rhythmic contractility and total work. Time, 30 second intervals. Top line, respiratory movements.

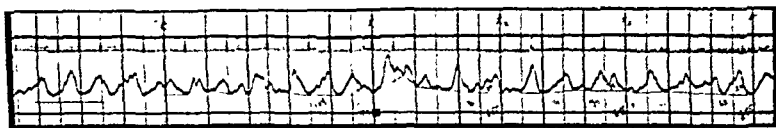


FIG. 4. EFFECT OF PYRIBENZAMINE (0.25 MG. PER KG. OF BODY WEIGHT)

See text for analysis of this record for the percentage increase of tone, rhythmic activity, and total work following the injection made at the time indicated by the time signal. Top, maternal respiratory movements. Time, 30-second intervals.

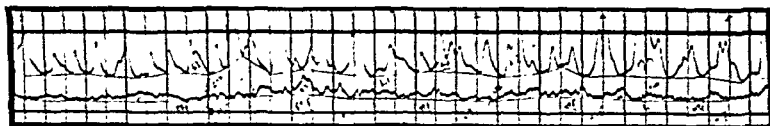


FIG. 5. SIMULTANEOUS RECORDINGS OF THE EFFECT OF PYRIBENZAMINE (0.5 MG. PER KG. OF BODY WEIGHT) AT A DISTENDED CONCEPTUS-SITE (INTRA-AMNIOTIC SAC) AND A NON-DISTENDED PORTION OF THE SAME UTERUS

The upper, more active record is made from the non-distended portion. The lower record is that of the conceptus site. See text for analysis (Table 3) showing percentage changes similar for both records, despite appearances. The topmost lead records maternal respiration but it cannot be read in this half-tone reproduction.

Respiration is recorded by means of an intrapleural trocar connected to another Statham gage dynamometer. In some experiments, intracarotid pressures have also been recorded.

PROCEDURES. After preparation of the animal and apparatus, a record of the normal activity for the conditions of recording is obtained. Customarily this is for one-half hour. A needle having a stylus in it is inserted into a marginal ear vein and clamped in place (see figure 1). The stylus is removed and a syringe attached to the needle at the time of injection. The duration of the injection is recorded by a signal (see figures 3, 4, and 5). Time intervals are marked by a line across the paper at thirty second intervals. The time line serves also as the

base-line for the uterine tracings. The paper speed is about 2.15 centimeters per minute.

Injections are not repeated for one-half hour or until a long record of uterine activity comparable to that in the control period is obtained. At the termination of the experiment, the loop of uterus is dissected free from the mesometrium and other tissues and weighed. Planimeter measurements of enlarged drawings of cross sections of uteri show that the myometrium of the non-gravid uterus or of the undistended portion of the gravid uterus comprises approximately 65% of the tissue. The number of grams of myometrium contributing to the record of activity is estimated, accordingly, as this fraction of the wet weight of tissues in any loop of uterus used.

Calculations. Analysis of records of the sort shown in figures 3, 4, and 5 is made for two purposes. In one, the relative change in uterine activity over a control, or pre-injection period is determined for given periods of time following the injection. In the other, the data are analyzed for the force developed per gram of myometrium. The former is expressed as a percentage change. The latter is calculated in terms of dynes per gram of muscle per minute. In both calculations, the records are analyzed for work-done-as-tone and work-done-as-active-contractions over and above tone. Both types of calculation depend upon the same initial reading of the record. Consequently, the manner of doing this will be described first.

(1) *Reading the records.* The method of marking the records is shown in figures 3, 4, and 5. A line is drawn connecting the bases of the major rhythmic contractions. The record is then blocked off in periods before and after injection, beginning with the nearest half-minute line before or after the signal for the injection. Convenient time periods for purposes of analyzing the effect of anti-histaminic drugs are as follows; for the control, a five-minute period; for the duration of the drug effect, three-minute periods. These are marked by the symbols at the top of each record in figures 3, 4, and 5.

The zero correction for the element recording uterine activity is determined by noting the distance between the true zero (obtained as described above, by the recording of atmospheric pressure) and the injection-signal, which appears as a straight base-line throughout the record.

The observed area, defined as that area bounded by the base-line at the bottom, the time signals at the beginning and the end of a period and the curve of uterine activity, is measured by means of a planimeter. Correction for zero to arrive at a true area is then made. This area is proportional to the total work done during that particular period. The area between the line connecting the bases of the contractions and the base-line less the correction for zero within any period is proportional to the work involved in maintaining muscle tone, and the difference between these two areas represents work due to activity resulting from uterine contractions superimposed on tone.

(2) *Relative changes in uterine responsiveness* are measured by dividing the true areas by the number of minutes in each experimental period and expressing this as a percentage increase or decrease from the control period. This may be done for tone, activity over tone, or for total activity.

(3) Rate of work may be calculated in the following three steps:

(a) The true area in square centimeters (A_t) is equal to the observed area (A_{obs}) less the zero correction in centimeters (0_{cm}) times the length in centimeters of the baseline (L_{cm}). This relationship may be expressed as,

$$A_t = A_{obs} - (0_{cm} \times L_{cm})$$

(b) The total force, in dynes (F_d), exerted throughout a given period is equal to the true area times the force of the deflection in terms of cubic centimeters of water pressure (P_{cc}) per centimeter of deflection on the paper for the gage and element used (Cm_D) and times the number of seconds elapsed per centimeter of photographic paper traveled (S/cm). This relationship may be expressed as,

$$F_d = A_t \left(\frac{P_{cc}}{Cm_D} \right) \times \left(\frac{S}{cm} \right)$$

(c) The rate of work in dynes per gram of myometrium per minute ($F_d/gm./min.$) is equal to the total dynes (F_d) divided by the duration of the period in minutes (t) and by the product of the weight of the loop of uterus in grams (W_u) and the factor 0.65 (the proportion of myometrium in the uterus). This may be expressed as,

$$\text{Rate of Work} = F_d/gm./min. = \frac{\left(\frac{F_d}{t} \right)}{(W_u \times 0.65)}$$

RESULTS. The results to be described in this paper will be presented in three parts. First, an analysis will be given of the rate of work of the uterus following administration of 1.5 minims of Pitocin intravenously. Second, an example will be presented which deals with the relative, or percentage changes following injection of the anti-histaminic drug, tripolamine (Pyribenzamine). Third, a brief summary of the general effects of Pyribenzamine and diphenhydromine (Benadryl) upon the uterus of the rabbit will be given.

RATE OF WORK OF THE UTERUS FOLLOWING PITOCIN. An analysis of the record shown in figure 3 is given below. In this experiment, a non-pregnant rabbit weighing 3.6 kilograms was prepared as outlined above. The ovaries contained a number of medium-sized follicles and numerous old corpora lutea. The uterus was pink and actively motile. The cornual end of the right uterus was used.

With the gage and recording element used, a deflection on the paper of 1 centimeter was equal to 16.0 centimeters of water pressure, or 16.0 cubic centimeters of water pressure per square centimeter of surface (P_{cc}). At the point indicated by the signal, Pitocin was injected. The analysis covers a control period immediately preceding, and three three-minute periods following the injection. This is summarized in table 1.

We see in table 1 (d) that by far the greatest effect of Pitocin in this experiment was in the effect on tone. The effect on rhythmic contractions was limited

TABLE 1

*Rate of work of the work of the uterus following Pitocin*a. Areas (A_{obs}) uncorrected, written on the record immediately after planimetric determination

	LENGTH OF TIME LINE (L_{cm})	TOTAL AREA (CM^2)	AREA FOR TONE (CM^2)	AREA FOR ACTIVITY (CM^2)
Control 5 min. . .	10.80	12.00	8.10	3.90
Period 1-3 min.	6.40	11.00	8.10	2.90
Period 2-3 min.	6.55	7.30	5.30	2.00
Period 3-3 min.	6.45	7.30	5.50	1.80

The correction for true zero in this record is -0.13 centimeter (0_{cm}).b True areas (A_t) in square centimeters per minute, including the zero correction

	TOTAL (A_t/MIN)	TONE (A_t/MIN)	ACTIVITY (A_t/MIN)
Control	10.59	6.69	3.90
Period 1	10 16	7.26	2.90
Period 2	6.44	4.44	2.00
Period 3	6 46	4.66	1 80

c. Energy output in dynes for each period

(1) Deflection of 1 centimeter (CM_D) equals 16 centimeters of water pressure, or 16 cubic centimeter of water pressure (P_{cc}).(2) One square centimeter on the paper moving 28 seconds/cm (S/cm) is equal to 448 dynes (F_d).

(3) Dynes output for each period of the experiment

	TOTAL WORK (F_d)	TONE WORK (F_d)	ACTIVITY WORK (F_d)
Control	4750	3000	1750
Period 1	4550	3250	1300
Period 2	2880	1990	896
Period 3	2897	2090	807

d. The weight of the loop of uterus used was 0.75 gms. (W_u). Since 65% of this is myometrium (see text), the weight of myometrium performing the work is 0.5 gms. The work output per gram per minute ($F_d/gm/min$) is as follows

	RATE OF TOTAL WORK ($F_d/gm/min$)	RATE OF TONE WORK ($F_d/gm/min$)	RATE OF ACTIVITY WORK ($F_d/gm/min$)
Control	1900	1200	700
Period 1	3032	2166	866
Period 2	1923	1326	597
Period 3	1930	1392	538

to the first three minutes, after which the activity was substantially less than during the control period. This conclusion would be difficult to make from inspection of the record alone.

RELATIVE EFFECTS OF AN ANTI-HISTAMINIC DRUG ON THE UTERUS. An example of the effect of Pyribenzamine on the uterus is shown in figure 4. The rabbit employed in this experiment weighed 3.6 kilogram. The ovaries contained medium and large sized follicles, but no corpora lutea. The zero correction was 0.2 centimeters. At the signal, 0.25 milligrams per kilogram of Pyribenzamine was injected.

TABLE 2
Effect of Pyribenzamine
1) Observed areas

	L_{cm}	tone (cm ²)	ACTIVITY (cm ²)	TOTAL (cm ²)
Control . . .	10.9	104	47	147
Period 1	6.55	81	39	120
Period 2 . . .	6.45	70	38	108
Period 3	6.45	63	26	89

$0_{cm} = 0.20$.

2) True area per minute (A_t/min)

	tone (cm ²)	ACTIVITY (cm ²)	TOTAL (cm ²)
Control . . .	20.36	9.50	29.86
Period 1	26.56	13.00	39.56
Period 2	22.90	12.66	35.56
Period 3	20.57	8.66	29.23

3) Percentage change after Pyribenzamine

	tone	ACTIVITY	TOTAL
	%	%	%
Control . . .	100	100	100
Period 1	130.4	136.8	132.5
Period 2	112.5	133.2	119.1
Period 3	101.0	91.1	97.9

As in the case of the previous example, the observed areas are marked in their respective periods. In table 2, the steps taken to convert these to true areas, corrected for the zero error, as above, are shown, along with the percentage changes in square centimeters per minute due to tone, square centimeters per minute due to activity, and the total areas. These areas are proportional to the work per minute, since each square centimeter of recorded activity equaled 448 dynes in this experiment.

Inspection of the record in figure 4 suggests that Pyribenzamine brought about a transient period of slightly lessened activity after the initial large effect immedi-

ately following the injection. The analysis of this record summarized in table 2 shows quite a contrary effect, however, with respect to tone, and the decrease in total activity in the third period probably was not significant (only 2% less than during the control).

OBSERVATIONS ON THE EFFECTS OF PYRIBENZAMINE AND BENADRYL. In this work, many rabbits have been employed from time to time in testing the effects of prostigmine, acetylcholine, histamine, posterior pituitary substances, cholinesterase, di-isopropylfluorophosphate and anti-histaminic drugs. The final method, with data sufficient for analysis was applied mostly with respect to anti-histaminic drugs, however. In all, twenty-two rabbits were used, with one to six injections made in each experiment. Qualitatively, oxytocic responses were obtained in every instance, such as is seen in the second example above.

Certain features of the responses are worth noting, however, since observations on the uterine action of anti-histaminic drugs are few and scattered (2, 3). The

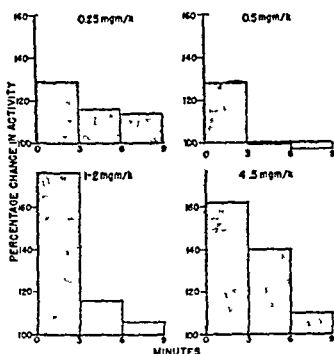


FIG. 6. SUMMARY OF THE ACTION OF DIFFERENT DOSES OF PYRIBENZAMINE ON THE MYOMETRIUM

effects will be considered with respect to the action of different doses; the effect of distention of the uterus by the products of conception; and the effect of hormonal status of the animal on the response to Pyribenzamine and Benadryl.

Effect of dose on uterine response. The effects of Pyribenzamine and of Benadryl are shown in figures 6 and 7. All the results at different dose levels are combined for the two drugs.

In the case of each drug, an oxytocic effect was obtained at all dose levels during the first three-minute period after injection. What occurs in the next three to six minutes depends, apparently, upon the drug and the dose. Oxytocic effects were obtained at this time with Pyribenzamine except at the level of 0.5 milligrams per kilogram of body weight. With this dose, activity returned to about normal levels by the second period. With Benadryl, definitely less than normal activity resulted at the same dose level, and at 0.25 milligrams per kilogram of body weight, definitely less than normal activity was observed in the third period. At the lower dose levels, therefore, Benadryl appears to be a more

effective sedative of uterine activity than Pyribenzamine after an initial oxytocic effect.

The larger dose (1-2 milligrams per kilogram of body weight) has a greater oxytocic effect during all three periods following injection. Benadryl is more highly oxytocic than Pyribenzamine in higher doses.

Effects on tone and rhythmic activity. The effects of Pyribenzamine and Benadryl on tone and rhythmic contractility of the myometrium are shown in figure

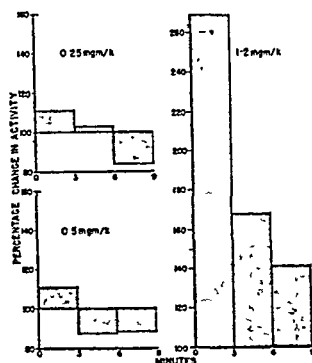


FIG. 7. SUMMARY OF THE ACTION OF DIFFERENT DOSES OF BENADRYL ON THE MYOMETRIUM

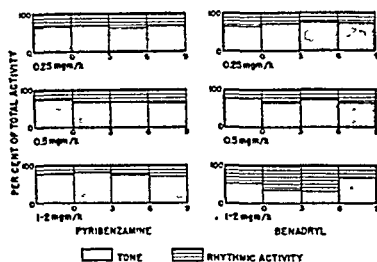


FIG. 8. EFFECT OF PYRIBENZAMINE (LEFT) AND BENADRYL (RIGHT) ON THE PROPORTION OF THE TOTAL ACTIVITY ATTRIBUTABLE TO TONE AND TO RHYTHMIC ACTIVITY, FOR THREE THREE-MINUTE PERIODS FOLLOWING THE INJECTION

8. Here, it will be noted, there is no appreciable change in the proportion of activity due to tone and rhythmic activity respectively in any three-minute period following injection, with the exception of the larger doses of Benadryl. The oxytocic effect in this instance is attributable to a disproportionate increase in rhythmic contractility, although there was also an increase in the absolute tone following the injection.

Effects of the products of conception. As mentioned above, and as shown in figures 2 and 5, some experiments have been carried out with simultaneous recordings from within the amniotic sac in the uterus of pregnant rabbits and

from non-distended portions of the gravid uterus. In figure 5, a rabbit weighing 3.25 kilograms received 0.5 milligrams of Pyribenzamine per kilogram of body weight on the 18th day. If one looks at the curves of each of these tracings, it appears that that portion of the uterus which is distended by the products of conception is not much affected by Pyribenzamine, whereas the non-distended part of the gravid uterus is highly reactive. Analysis of these two curves leads one to a different conclusion, however.

TABLE 3

Percentage changes in tone and rhythmic activity of the gravid uterus following Pyribenzamine in (a) non-distended part of the uterus and (b) in the distended part (amniotic sac)

	TONE		RHYTHMIC ACTIVITY		TOTAL	
	Non-distended	Distended	Non-distended	Distended	Non-distended	Distended
	%	%	%	%	%	%
Control. .	100	100	100	100	100	100
Period 1	118.1	157.5	171.9	135.7	131.0	150.4
Period 2.	105.6	104.9	129.0	117.6	111.4	108.9
Period 3	108.9	142.6	129.0	117.6	115.4	134.3
Period 4	98.9	127.6	185.1	92.6	119.8	116.1

TABLE 4

Effect of hormonal status on activity of the uterus following injection of Pyribenzamine
Results expressed as percentage increase in activity of that in the control period

Doses mgm./k.	TONE		RHYTHMIC ACTIVITY		TOTAL ACTIVITY	
	Non-preg.	Preg.	Non-preg.	Preg.	Non-preg.	Preg.
0.25	130	112	133	111	132	127
0.50	112	118	153	162	123	130
1-2	114	122	204	380*	140	188
4-5	194	136	174	186	192	153

* Attributable largely to one very high percentage increase (620%).

The percentage changes in activity of the two curves after injection are summarized in table 3. Here, it will be seen that a greater percentage increase in total work was observed at the site of distention by the products of conception than in the non-distended region. The two components, tone and activity are affected differently. It should be noted, however, that the initial pressure in the amniotic sac was about 3.4 centimeters of water, and about 6.9 centimeters of water in the non-gravid portion of the uterus.

Effects of hormonal status on uterine response. Our data are too few to offer more than suggestive evidence concerning the effect of the hormonal status of the rabbit upon the uterine response to anti-histaminic drugs. Not enough

pregnant and non-pregnant rabbits were used at all dose levels to make possible a statistical solution of this problem. Even so, the data seem conclusive on several points. All pregnant rabbits used were between the twelfth and eighteenth days of pregnancy. Consequently data were obtained during the period of a high level of function of the corpora lutea of pregnancy. Since there is less variation in the first three-minute period following injection of Pyribenzamine than later, comparison will be made only for this period in non-pregnant and pregnant rabbits, respectively.

Table 4 contains a summary of the effect of Pyribenzamine during the first three-minute interval following the injection. It is clear from this that, so far as these data go, there is no clear effect of hormonal status of the recipient at the time of the injection either on the magnitude or the quality of the result obtained. At each dose level considered, the percentage of increased activity is of the same order. This is generally true when the effects on tone and activity are considered as well as total activity particularly with respect to the two lower doses employed in this study. Thus it appears that the effect of Pyribenzamine upon the myometrium of the rabbit is not influenced by hormonal status at the time of injection.

SUMMARY

1. The method of using a strain gage dynamometer for quantitative study of pharmacological responses of the myometrium is described.
2. The method permits determination of the effect on tone and rhythmic contractility.
3. Results are obtained either as, (a) the percentage increase in activity (tone, contractility, total work), or (b) as dynes per gram of myometrium per minute (for tone, contractility, and total work).
4. Quantitative effects of Pitocin, Pyribenzamine, and Benadryl are cited.
5. The effect of the anti-histaminic drugs administered intravenously is oxytocic, the effect being greater with increased doses. With low doses, a period of diminished activity may follow the initial oxytocic response.
6. Tone and rhythmic contractility are equally affected by the anti-histaminic drugs.
7. The uterus distended by the products of conception is affected in a manner similar to the non-distended portion of the uterus of the same animal.
8. The hormonal status (pregnancy, non-pregnancy) of the rabbit does not affect the responsiveness of the uterus to Pyribenzamine.

REFERENCES

1. MEYER, R. D.: *Instruments*, 19: 139, 1946.
2. LOEW, E. R.: *Physiol. Rev.*, 27: 542, 1947.
3. SHERROD, T., LOEW, E. R., AND SCHLAEMER, H.: *THIS JOURNAL*, 89: 247, 1947.

from non-distended portions of the gravid uterus. In figure 5, a rabbit weighing 3.25 kilograms received 0.5 milligrams of Pyribenzamine per kilogram of body weight on the 18th day. If one looks at the curves of each of these tracings, it appears that that portion of the uterus which is distended by the products of conception is not much affected by Pyribenzamine, whereas the non-distended part of the gravid uterus is highly reactive. Analysis of these two curves leads one to a different conclusion, however.

TABLE 3

Percentage changes in tone and rhythmic activity of the gravid uterus following Pyribenzamine in (a) non-distended part of the uterus and (b) in the distended part (amniotic sac)

	TONE		RHYTHMIC ACTIVITY		TOTAL	
	Non-distended	Distended	Non-distended	Distended	Non-distended	Distended
	%	%	%	%	%	%
Control.....	100	100	100	100	100	100
Period 1	118.1	157.5	171.9	135.7	131.0	150.4
Period 2.	105.6	104.9	129.0	117.6	111.4	108.9
Period 3.. . . .	108.9	142.6	129.0	117.6	115.4	134.3
Period 4	98.9	127.6	185.1	92.6	119.8	116.1

TABLE 4

Effect of hormonal status on activity of the uterus following injection of Pyribenzamine
Results expressed as percentage increase in activity of that in the control period

Doses mgm./k.	TONE		RHYTHMIC ACTIVITY		TOTAL ACTIVITY	
	Non-preg.	Preg.	Non-preg.	Preg.	Non-preg.	Preg.
0.25	130	112	133	111	132	127
0.50	112	118	153	162	123	130
1-2	114	122	204	380*	140	188
4-5	194	136	174	186	192	153

* Attributable largely to one very high percentage increase (620%).

The percentage changes in activity of the two curves after injection are summarized in table 3. Here, it will be seen that a greater percentage increase in total work was observed at the site of distention by the products of conception than in the non-distended region. The two components, tone and activity are affected differently. It should be noted, however, that the initial pressure in the amniotic sac was about 3.4 centimeters of water, and about 6.9 centimeters of water in the non-gravid portion of the uterus.

Effects of hormonal status on uterine response. Our data are too few to offer more than suggestive evidence concerning the effect of the hormonal status of the rabbit upon the uterine response to anti-histaminic drugs. Not enough

one gave the most sedation in the subjects. Several of them slept between visits to the fluoroscope. Whether they slept or not also showed no correlation with the effects on motility.

Sodium pentobarbital in the dosage used produced significantly increased motility in six of the ten subjects, and the averages for the series as a whole showed a decrease in emptying time.

DISCUSSION. Gruber and Gruber (3) reported that large doses of barbiturates caused depression of gastric motility in fistula dogs. On the other hand, Dreyer et al. (4) found increased motility of the stomach and small intestine of the cat with small doses of barbiturates. They reported a rough parallelism between hypnotic activity and stimulating effect. A more extensive discussion of the literature is given in Van Liere and Northup (1).

The results obtained here indicate that the increased motility found with small doses does not extend to drugs used in this investigation other than the barbiturates, since in the dosages used paraldehyde had the greatest sedative and hypnotic effect, while pentobarbital had the greatest stimulating effect on

TABLE I

	NO. OF SUBJECTS	EMPTYING TIME IN HOURS	
		Control	Experimental
Chloral Hydrate, 33-60 gm. . . .	12	2.20	1.97
Pentobarbital, 100 mgm. . . .	10	2.22	1.85*
Bromural, .6 gm.	7	2.29	2.02
Paraldehyde, 6 cc.	14	2.02	2.02

* Significant at the 5% level (Fisher's "t" test).

the motility of the stomach. This fact, in conjunction with the previous findings on sodium amytal, indicates that the effect is due to something other than sedation, and since significant changes in any particular series were found only with the barbiturates, it appears to be some action which is present to a greater degree in them than in the other compounds investigated. The mechanism of this action is not apparent from the data available.

SUMMARY

The effect of therapeutic doses of chloral hydrate, bromural, pentobarbital sodium, and paraldehyde on the motility of the human stomach was studied. Consistently increased motility was found only with pentobarbital sodium, indicating that sedation is not the principal factor responsible for the decreased emptying time seen with certain central nervous system depressants (barbiturates).

REFERENCES

1. VAN LIERE AND NORTHUP: THIS JOURNAL, 73: 142, 1941.
2. NORTHUP AND VAN LIERE: Federation Proceedings, 5: 77, 1946.
3. GRUBER AND GRUBER: THIS JOURNAL, 72: 126, 1941.
4. DREYER, RAY AND LARSON: Federation Proceedings, 3: 70, 1944.

THE EFFECT OF CENTRAL NERVOUS SYSTEM DEPRESSANTS ON THE EMPTYING TIME OF THE HUMAN STOMACH

DAVID W. NORTHUP AND EDWARD J. VAN LIERE

Department of Physiology, School of Medicine, West Virginia University, Morgantown

Received for publication March 2, 1948

In a paper previously published from this laboratory (1) it was shown that sodium amytal produced a decrease in the emptying time of the human stomach, as determined fluoroscopically with a farina test meal to which barium sulfate was added. The present study is an extension of this work to include other agents, including a similar barbiturate (pentobarbital sodium) and representatives of other chemical classes with similar actions, in an attempt to determine whether the effect is a general one, or is specific to one class of drugs. The other drugs used were chloral hydrate, bromural (2-monobromisovalerylurea), and paraldehyde. In each case therapeutic doses were employed; the subjects were medical students.

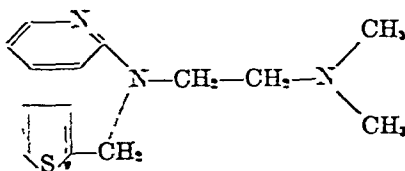
The results obtained with chloral hydrate have been previously published in a preliminary report (2).

METHODS. The test meal used in other studies from this laboratory was used. This consisted of 15 grams of Quaker Farina, boiled in water with one gram of salt, to a volume of 200 cc.; 100 grams of barium sulfate were added to make the mixture visible in the fluoroscope. Determinations of emptying times, either control or experimental, were made not oftener than once a week. When drugs were administered, they were given orally immediately before the meal was eaten. Gastric emptying time was determined to the nearest fifteen minutes. At least three control and three experimental determinations were done on each subject.

RESULTS. The results are summarized in Table I. Chloral hydrate was given in two different dosages, .33 gm. and .60 gm. Neither dosage gave a significant change in emptying time. Shown in the table are the averages for the combined series, which also showed no significant change. It is interesting, however, that when the figures for each subject are submitted to statistical analysis by Fisher's "t" test, three of the twelve subjects showed decreases in emptying time (increased motility) which were significant at the 5% level or better.

The bromural series as a whole did not show any significant change, but here again one subject had a significantly shortened emptying time—from an average of 2.45 hours in the control to 1.80 hours after 0.6 gm. of bromural. None of the other subjects showed such a marked effect, and one was slightly, but not significantly, delayed after administration of the drug.

The averages for the paraldehyde series as a whole showed absolutely no change, but the emptying times for five of the subjects showed significant alterations; three were speeded up and two were slowed. Some of the subjects were nauseated briefly by the drug, but this showed no correlation with the changes in emptying time. It is interesting to note also that of all the drugs used, this



W-53

The low relative, as well as the absolute, toxicity of W-50 and its high therapeutic index led us to recommend this preparation for clinical trial. The results of clinical investigation are in agreement with our pharmacological findings: the compound is reported by Combes (8) to possess the advantage of a high tolerance in man.

The present publication contains the pharmacological and toxicological studies conducted on this antihistaminic.

EXPERIMENTAL PART

(I) Antihistaminic activity

(1) Activity on isolated organs.

(a) The contraction of the isolated guinea pig intestine induced by 1–2 γ /cc histamine was inhibited by dilutions of Diatrin varying from 1:1,000,000 to 1:10,000,000 (final concentration in the tissue bath, at 37°C). The same antihistaminic content was required for the 4 compounds used for comparison.

(b) The isolated uterus of guinea pig required 1 γ /cc histamine for contraction. This histamine effect was inhibited by Diatrin, 1:1,000,000.

(c) The isolated lung of the guinea pig was prepared for perfusion as described by Tainter et al (9). The addition of 20 to 50 γ of histamine reduced to half the volume of the perfusion fluid passing through the lung. This effect of histamine, determined by bronchial contraction, was prevented by the addition of Diatrin in 0.5–1.0 mg doses.

(2) Protection against intravenously injected histamine.

The antihistaminic agent was administered subcutaneously to guinea pigs weighing 150–250 grams. After this protective treatment, various multiples of the lethal histamine dose—0.5 mg/kg histamine dihydrochloride—were injected into the saphenous vein. The minimal dose of Diatrin which protected guinea pigs against 2–5 lethal doses of histamine was 0.25–0.50 mg/kg (Table 1).

Guinea pigs injected with 100 to 200 times the lethal dose of histamine required correspondingly higher doses of Diatrin for protection. Ten out of 12 animals injected subcutaneously with 25–40 mg/kg of Diatrin survived the injection of 100 lethal doses of histamine given intravenously. The protection was effective only against acute histamine shock; the gastric ulceration which followed within 5–18 hours could not be prevented. This finding is in agreement with our own experience with other antihistaminics, and with the reports of Friesen et al (10), Winter (11), and Halpern (12a).

THE TOXICOLOGIC AND ANTIHISTAMINIC PROPERTIES OF N,N'-DIMETHYL-N'-PHENYL-N'-(2-THIENYLMETHYL) ETHYLENE-DIAMINE HYDROCHLORIDE (DIATRIN)

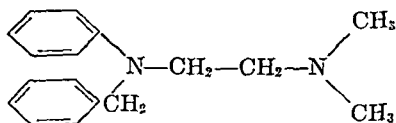
N. ERCOLI, R. J. SCHACHTER, W. C. HUEPER AND M. N. LEWIS

Department of Pharmacology and Chemotherapy, Warner Institute for Therapeutic Research, New York 11, N. Y.

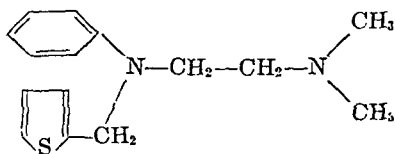
Received for publication March 2, 1948

Definite limitations in the clinical use of the synthetic antihistaminics recently introduced in therapy result from the high incidence of side effects encountered and from the potential danger of untoward reactions (1-3). The pharmacological mechanism of these untoward reactions (excitement, depression, etc.) appears to be related mainly to the action of these drugs on the central nervous system. In animal experiments, the toxic manifestations consist of spasticity, excitement, convulsions, depression, drowsiness, salivation and vomiting. These symptoms appear in different sequence and degree according to the animal species, the particular compound (Benadryl, Pyribenzamine, Neo-antergan, etc.), and the dosage used.

Undoubtedly there is a relationship between the toxic doses and manifestations in animal experiments and the severity and incidence of clinical side effects; it may be assumed that a drug with lower toxicity and less marked action on the central nervous system in experimental animals will be better tolerated clinically also. Among a series of thenyl derivatives of ethylenediamine synthesized in this Institute by Leonard and Solmssen (4), one preparation attracted our attention as promising from this standpoint. This compound, N,N'-dimethyl-N'-phenyl N'-(2 thienylmethyl) ethylenediamine hydrochloride (Diatrin hydrochloride), is an analogue of Antergan and of W-53, another thenyl derivative which already has been reported to possess definite antihistaminic properties (5-7).



Antergan



Diatrin (W-50)

the surrounding skin. In animals treated with Diatrin, this phenomenon was *qualitatively* changed or abolished. In rabbits treated parenterally with 2 mg/kg, the phenomenon of the "blue ring" was suppressed; instead, the coloration appeared within 1-4 minutes in the interior of the injected zone. In-

TABLE 2
Protective activity of Diatrin against lethal histamine asthma

DOSE INJECTED	NUMBER OF ANIMALS	NUMBER DIED	% SURVIVORS
Intramuscular Injection			
mg/kg			
—	27	22	18.5
0.025	1	0	
0.05	5	2	60
0.10	20	9	55
0.20	3	0	100
0.3-0.5	3	0	100
Subcutaneous Injection			
—	25	22	12
0.05	12	5	58
0.10	14	7	50
0.20	10	3	70
0.5-1.0	3	0	100

TABLE 3
Effect of Diatrin on histamine hypotension

DIATRIN I.V.	HISTAMINE IN γ	DROP OF BLOOD PRESSURE IN MM/HG					
		Before Treatment	After treatment				
			5 min.	$\frac{1}{2}$ hr.	$\frac{1}{2}$ hr.	1 hr.	2 hrs.
mg/kg							
1 (dog)	1	14	0			*	12
	2	18	8			8	16
	4	27	11				
8 (cat)	0.5	36		0	0		0
	Acetylcholine						
	0.5 γ	44		45	45		45

* Not measurable.

creasing the dose of Diatrin to 5-8 mg/kg, the uptake of the dye by the histamine zone became negligible, and/or greatly delayed. These observations, indicating that antihistaminics might possibly affect the histamine action on the skin by different mechanisms, deserve further study. An interference with wheal formation by Benadryl and Neo-antergan has been described by Last and Loew (15).

(3) *Protection against lethal histamine asthma.*

The method described by Halpern (12b) and by Loew, Kaiser and Moore (13) was used. In each experiment one untreated and 2-3 treated guinea pigs were exposed to histamine inhalation. Untreated animals generally died with convulsions after 2-3 minutes' exposure. The effect of treatment, which consisted in either alleviation of symptoms or protection against death, is given in Table 2. The minimal active dose of Diatrin against lethal amounts of inhaled histamine was 0.05 mg/kg given intramuscularly or subcutaneously.

TABLE 1

Activity of antihistamines against intravenous lethal histamine doses in guinea pigs

SUBCUTANEOUS DOSE mg/kg	DIATRIN				BENADRYL				W-53				PYRIBENZAMINE			
	2 LD		5 LD		2 LD		5 LD		2 LD		5 LD		2 LD		5 LD	
	conv.	death	conv.	death	conv.	death	conv.	death	conv.	death	conv.	death	conv.	death	conv.	death
0.01													4/4	3/4		
0.025									2/2	2/2			6/6	3/6		
0.05									2/3	0/3	3/3	3/3	4/7	0/7	4/5	4/5
0.10	6/6*	6/6							0/1	0/1	4/4	0/4	0/2	0/2	0/7	1/7
0.25	5/7	3/7	3/3	3/3†	4/4	2/2			0/1	0/1	1/1	0/1	0/1	0/1	1/3	0/3
0.50	0/6	0/6	7/7	4/7	5/7	1/7	3/3	2/3	0/1	0/1	1/1	0/1				0/1
1.0		0/2	5/5	2/5			5/5	3/5								
2.0			5/7	1/7			5/7	0/7								

2 LD = treatment followed by 2 lethal intravenous histamine doses.

5 LD = treatment followed by 5 lethal intravenous histamine doses.

* number animals reacting with convulsion/number animals treated.

† number died/number treated.

(4) *Influence on the blood pressure lowering effect of histamine.*

The carotid blood pressure of dogs in anaesthesia (sodium phenobarbital 120-160 mg/kg, or pentobarbital 35 mg/kg intraperitoneally) was recorded on a kymograph. Various doses of histamine (0.5-4 γ) were injected intravenously before and after intravenous Diatrin treatment. Doses above 0.5 mg/kg reduced or blocked the hypotension induced by histamine for more than 2 hours.

(5) *Influence on the histamine wheal.*

The method described by Rocha e Silva and Dragstedt (14) was used to visualize the effect of histamine, or its inhibition, on the skin. Volumes of 0.2 cc histamine 1:1000 and 1:10,000 were injected intradermally on the shaved backs of albino rabbits. Immediately after injection, 1 cc/kg of a 1% Trypan blue solution was injected intravenously into the ear vein. In the untreated rabbit, $\frac{1}{4}$ to 1 minute after the injection of the dye, a blue ring appeared in the zone of the injected histamine; this ring became increasingly colored; within 1-5 minutes a (full) blue circle, corresponding to the histamine wheal, formed a contrast to

studied comparatively. The tissues of animals used for this investigation were subjected to histologic examination.

(1) *Acute toxicity.*

The results of the experiments on the acute toxicity of the various antihistaminic drugs tested are summarized in Table 6.

Symptoms. In mice, distinct differences were noted in the length of time which elapsed between the administration of the various antihistaminics and the onset of convulsive symptoms. The duration of the convulsive states elicited by comparative doses of the different antihistaminics varied. The tonicoclonic,

TABLE 5

Effect of antihistaminics on circulation and respiration by intravenous injection in dogs

DRUG	DOSE MG/KG	PULSE RATE VARIATION/MIN.	RESPIRATORY RATE VARIATION/MIN.	BLOOD PRESSURE VARIATION MM/HG	RETURN TO NORMAL AFTER MINUTES
Diatrin	0.2	+10, +0	0, -1	0, +5	1, 1*
	1.0	0, -10, -16, -4, -8	+4, -7, 0, 0, -1	-24, 0, -4, -30, -37	3, 1, 2, 9
	2.0	4, -12, -14, -8	+4, +1, +6, +6, +16	-54, -46, -48, -44, -52	3, 4, 5, 4, 4
	4-5	-12, +42, +28, -16, +2, -4	+7, +25, +12, +30, +21, +7, +36	-96, -49, -68, -60, -70, -64, -56	4, 7, 7, 12, 12, 7, 25
	8-10	-14, +30	+45, +39	-95, -60	15, 30
Pyribenzamine	0.2	+4	+1	+10	1
	1.0	-28, 0	+16, 0	0, +28, +10	8
	2.0	-12	+20, +8	-78, -30	12, 5
	4.5	0, +12	+39, +3	-110, -84	10, 12
Benadryl	0.2	-5	0	0	1
	1.0	+4	-1	+15	1

* Each figure corresponds to one injection of the drug.

epileptiform convulsions elicited by all drugs studied were of much longer duration in mice (1-2 hours) than in rabbits, in which they usually lasted no longer than 2-15 minutes. As a rule, convulsions in rabbits started one minute after the administration of the drug; only occasionally was the onset delayed by 10-15 minutes. The animals showed an increased tonicity or spasticity of the muscles for some time after the convulsions had ceased, and mechanical stimulation elicited a new convulsive attack during this period. Opisthotonos was present during these attacks, which were followed by a prolonged period of depression and general weakness.

The majority of the mice which succumbed to the effect of antihistaminics died during the prolonged convulsive stage. When very high lethal doses were given, death followed within a few minutes.

(6) *Activity in the anaphylactic shock of guinea pigs.*

Guinea pigs, sensitized 2 weeks before by the subcutaneous injection of 0.1 cc. horse serum, were injected *intravenously* with 0.2 cc of the same serum. Typical symptoms of anaphylactic shock, ending with death in 80% of the cases, followed this injection. In animals treated subcutaneously with 1.0 mg/kg Diatrin, only very mild signs—ruffled fur, sneezing, red ears—were induced by the second injection of serum and the animals survived (Table 4).

TABLE 4
Therapeutic activity of Diatrin in anaphylactic shock of guinea pigs
(Shocking dose used: 0.2 cc horse serum intravenously)

DRUG	DIATRIN	NO. PIGS	SYMPTOMS				DIED
			++++	++	±	0	
Controls	mg/kg —	30	27		3		24/30
Diatrin	0.125 i.v.	4	3	1			1/4
	0.25 i.v.	4		2	2		0/4
	0.5 s.c.	4	3	1			1/4
	1.0 s.c.	8	1	1	6		1/8
	2.0 s.c.	4		1	3		0/4
Pyribenzamine	0.25 s.c.	2	1		1		1/2
	0.50 s.c.	5	3		2		1/5
	1-2 s.c.	4	3		1		0/4
Non-sensitized controls*		3				3	0/3

* Injected with 0.5 cc serum.

++++ Severe convulsions, collapse.

++ Gasping or depression.

± Red ears, sneezing, ruffled fur.

0 No symptoms.

(II) *Pharmacologic experiments*

The effect of intravenous Diatrin on pulse, respiration and blood pressure was observed in 12 dogs and 6 cats under barbiturate anaesthesia. A drop in blood pressure of 24-37 mm followed the intravenous injection of 1 mg/kg Diatrin. Increased doses of Diatrin caused correspondingly lower blood pressure levels. The drop in blood pressure was accompanied, in general, by an increased respiratory rate, which was of slightly longer duration than the period of lowered blood pressure (4 minutes on the average). The changes in pulse were slight and variable even with doses as high as 5 mg/kg Diatrin. The pharmacological effect of Diatrin is similar to that of other antihistaminics (16, 17).

(III) *Toxico-pathological studies*

The acute and chronic toxicity of Diatrin, Pyribenzamine, Antergan, Neo-antergan, Benadryl, administered by various routes to different species, was

was a normal (12%) gain in weight during this period. None died from the effect of the medication. No gross pathologic changes were found at autopsy performed at the end of the experimental period. II. Groups of 10 rats (5 males, 5 females) were given, 5 times per week for 3 months, 20 mg/kg of the following antihistaminics: Diatrin, Pyribenzamine, Antergan, Neo-antergan, Benadryl. An eleventh group of 10 rats received, instead, distilled water for the same period. Body weight was determined every 2 weeks. The weight increases observed in the various series of rats fed with Diatrin were about the same as those seen in the control series. All animals were killed at the end of the experimental period.

The histopathologic study of the internal organs (brain, thyroid, thymus, lungs, heart, aorta, liver, pancreas, spleen, adrenals, kidneys, testes, ovaries, uterus, bladder, bone marrow) did not reveal any consistent and significant abnormalities. The majority of the rats of all series was free from any patho-

TABLE 7

Histologic lesions in rats after repeated oral administration of antihistamines
(group of 10 rats for each drug)

Per cent of animals affected.

SUBSTANCE	DIATRIN	PYRIBENZAMINE	ANTERGAN	NEO-ANTERGAN	BENADRYL	CONTROL
Lung Artery Hyalinosis	35	30	50	25	40	40
Brain degenerations	10	0	0	10	0	5
Testis degenerations	—	10	20	0	0	—
Liver degenerations	0	0	0	0	5	0

logic lesions. The brains of a few rats showed vacuolization of scattered ganglion cells, or a few pyknotic ganglion cells, or focal proliferations of glial cells. Medial hyperplasia and hyalinosis in the walls of small and medium sized pulmonary arteries, or subintimal calcified polyps in the pulmonary artery, were present in an appreciable number of rats, including those of the control series in which these changes were not quite as pronounced as in the experimental series. Testicular degenerations and focal liver cell regressions were observed in a few rats. The histologic findings are summarised in Table 7.

(b) *Subcutaneous administration.* 25 rats received, 6 times per week for 2 weeks, 25 mg/kg of Diatrin into the subcutaneous tissue of the back. All animals survived the experimental period without any apparent ill effects. There were no gross abnormalities found at autopsy.

(c) *Intravenous administration.* Groups of 6 rabbits each were given intravenously, 5 times per week for 6 weeks, the following antihistamines: Diatrin, Pyribenzamine, W-53, Neo-antergan and Benadryl. The initial dose was 8

Four dogs (average weight 10 kg) injected intravenously with 50 mg Diatrin exhibited only very mild signs of incoordination. Pyribenzamine given in the same dosage to 3 dogs induced severe convulsions, salivation, and, after the disappearance of these symptoms, spastic gait. Benadryl, in 2 dogs, gave only slight symptoms, such as spastic gait, lasting about 3 minutes.

In cats, (average weight $2\frac{1}{2}$ kg) a dose of 50 mg Diatrin given intravenously induced intense salivation in all 3 animals injected and vomiting in one (no convulsions). The same dose of Pyribenzamine caused violent convulsions. Benadryl caused convulsions in 2 out of 3 animals, while the third reacted with

TABLE 6

Comparative toxicity of Antihistaminics: Average convulsive (CD) and lethal doses (LD_{50}) in mg/kg

DRUG	SPECIES	NUMBER OF ANIMALS USED	INTRAVENOUS		SUBCUTANEOUS		ORAL	
			CD	LD_{50}	CD	LD_{50}	CD	LD_{50}
Diatrin	Mouse	739	25	45	125	160	450	550
	Rabbit	44	15	30				
	Guinea pig	128	15	30	50*	140	750	900
Pyribenzamine	Mouse	154	7	16	50	80	100	275
	Rabbit	19	5	10				
	Guinea pig	40			12.5	35		
Antergan	Mouse	158	10	35	75	125	150	325
	Rabbit	7	10	15				
Neo-Antergan	Mouse	197	17	25	75	115	200	325
	Rabbit	16	7	12				
Benadryl	Mouse	90			75	125	100	150
	Rabbit	14	10	12				
W-53	Mouse	73	8	17.5	40	75		

* Tolerated without convulsions: 40 mg/kg.

vomiting. Thus, Diatrin was better tolerated by dogs and cats than the antihistaminics used for comparison.

The data on the acute toxicity of Diatrin indicated that, depending on the route of administration, this substance is approximately $\frac{1}{4}$ to $\frac{1}{2}$ as toxic as the other five antihistaminic substances tested, as measured by the minimal convulsive doses and the LD_{50} . The LD_{50} obtained for the various antihistaminics and species in the present investigations is in general agreement with the data recorded by previous workers (18, 19).

(2) Chronic Toxicity.

(a) *Oral Administration.* I. Twenty-five rats of mixed sex were fed 75 mg/kg of Diatrin daily by stomach tube, 6 times per week, for 2 weeks. There

was a normal (12%) gain in weight during this period. None died from the effect of the medication. No gross pathologic changes were found at autopsy performed at the end of the experimental period. II. Groups of 10 rats (5 males, 5 females) were given, 5 times per week for 3 months, 20 mg/kg of the following antihistaminics: Diatrin, Pyribenzamine, Antergan, Neo-antergan, Benadryl. An eleventh group of 10 rats received, instead, distilled water for the same period. Body weight was determined every 2 weeks. The weight increases observed in the various series of rats fed with Diatrin were about the same as those seen in the control series. All animals were killed at the end of the experimental period.

The histopathologic study of the internal organs (brain, thyroid, thymus, lungs, heart, aorta, liver, pancreas, spleen, adrenals, kidneys, testes, ovaries, uterus, bladder, bone marrow) did not reveal any consistent and significant abnormalities. The majority of the rats of all series was free from any patho-

TABLE 7

Histologic lesions in rats after repeated oral administration of antihistamines
(group of 10 rats for each drug)

Per cent of animals affected.

SUBSTANCE	DIATRIN	PYRIBENZA- MINE	ANTERGAN	NEO- ANTERGAN	BENADRYL	CONTROL
Lung Artery Hy- alinosi s	35	30	50	25	40	40
Brain degenera- tions	10	0	0	10	0	5
Testis degenera- tions	—	10	20	0	0	—
Liver degenera- tions	0	0	0	0	5	0

logic lesions. The brains of a few rats showed vacuolization of scattered ganglion cells, or a few pyknotic ganglion cells, or focal proliferations of glial cells. Medial hyperplasia and hyalinosi s in the walls of small and medium sized pulmonary arteries, or subintimal calcified polyps in the pulmonary artery, were present in an appreciable number of rats, including those of the control series in which these changes were not quite as pronounced as in the experimental series. Testicular degenerations and focal liver cell regressions were observed in a few rats. The histologic findings are summarised in Table 7.

(b) *Subcutaneous administration.* 25 rats received, 6 times per week for 2 weeks, 25 mg/kg of Diatrin into the subcutaneous tissue of the back. All animals survived the experimental period without any apparent ill effects. There were no gross abnormalities found at autopsy.

(c) *Intravenous administration.* Groups of 6 rabbits each were given intravenously, 5 times per week for 6 weeks, the following antihistamines: Diatrin, Pyribenzamine, W-53, Neo-antergan and Benadryl. The initial dose was 8

Four dogs (average weight 10 kg) injected intravenously with 50 mg Diatrin exhibited only very mild signs of incoordination. Pyribenzamine given in the same dosage to 3 dogs induced severe convulsions, salivation, and, after the disappearance of these symptoms, spastic gait. Benadryl, in 2 dogs, gave only slight symptoms, such as spastic gait, lasting about 3 minutes.

In cats, (average weight $2\frac{1}{2}$ kg) a dose of 50 mg Diatrin given intravenously induced intense salivation in all 3 animals injected and vomiting in one (no convulsions). The same dose of Pyribenzamine caused violent convulsions. Benadryl caused convulsions in 2 out of 3 animals, while the third reacted with

TABLE 6

Comparative toxicity of Antihistaminics: Average convulsive (CD) and lethal doses (LD_{50}) in mg/kg

DRUG	SPECIES	NUMBER OF ANIMALS USED	INTRAVENOUS		SUBCUTANEOUS		ORAL	
			CD	LD_{50}	CD	LD_{50}	CD	LD_{50}
Diatrin	Mouse	739	25	45	125	160	450	550
	Rabbit	44	15	30				
	Guinea pig	128	15	30	50*	140	750	900
Pyribenzamine	Mouse	154	7	16	50	80	100	275
	Rabbit	19	5	10				
	Guinea pig	40			12.5	35		
Antergan	Mouse	158	10	35	75	125	150	325
	Rabbit	7	10	15				
Neo-Antergan	Mouse	197	17	25	75	115	200	325
	Rabbit	16	7	12				
Benadryl	Mouse	90			75	125	100	150
	Rabbit	14	10	12				
W-53	Mouse	73	8	17.5	40	75		

* Tolerated without convulsions: 40 mg/kg.

vomiting. Thus, Diatrin was better tolerated by dogs and cats than the antihistaminics used for comparison.

The data on the acute toxicity of Diatrin indicated that, depending on the route of administration, this substance is approximately $\frac{1}{4}$ to $\frac{1}{2}$ as toxic as the other five antihistaminic substances tested, as measured by the minimal convulsive doses and the LD_{50} . The LD_{50} obtained for the various antihistaminics and species in the present investigations is in general agreement with the data recorded by previous workers (18, 19).

(2) Chronic Toxicity.

(a) *Oral Administration.* I. Twenty-five rats of mixed sex were fed 75 mg/kg of Diatrin daily by stomach tube, 6 times per week, for 2 weeks. There

The histologic examination of the organs of the rabbits of both series repeatedly injected with antihistamines (intravenously) included brain, thyroid, thymus, heart, aorta, lungs, liver, pancreas, stomach, intestine, spleen, adrenals, kidneys, bladder, testes, ovary, uterus, bone marrow, lymph nodes, vena cava inferior. Minor and scattered degenerative changes in ganglion cells and glial cell foci were found in the brains of several rabbits given high doses of Diatrin, Pyribenzamine, Neo-artergan, and Benadryl. Medial thickenings and hyalinosis of the medium-sized and small pulmonary arteries were found in rabbits which received the high, almost lethal, doses of Diatrin, Pyribenzamine, Antergan, Neo-antergan and Benadryl. They were absent in all 6 rabbits to which 8 mg/kg of Diatrin were given repeatedly. Medial degenerations and calcifications of the aorta were present in some of the animals of the high dose groups given Diatrin, Pyribenzamine and Benadryl. Small degenerative foci and fibroblastic proliferations of the myocardium were noted in one or two rabbits of all groups. The same applied to the foci of fatty or hyaline degeneration of the liver, and the atrophic changes in the testes.

Thus, the pathologic lesions observed after the repeated oral and intravenous administration of large amounts of Diatrin to rats and rabbits were of relatively minor nature and appeared only when toxic sublethal doses were given repeatedly.

(3) *Detoxification.*

Fractions of the lethal doses were injected at various intervals, in order to determine the persistence of Diatrin in the organism. In a series of 28 rabbits injected intravenously with $\frac{1}{3}$ – $\frac{1}{2}$ the lethal dose, 10 and 15 mg/kg, at 10 to 30 minute intervals, the cumulative toxic effect appeared very clearly. The mortality rate decreased when the interval between injections was increased to 1 hour. These results indicate that the detoxification or excretion of Diatrin in the organism takes place at a relatively rapid rate.

Attempts to counteract the toxic effect of antihistaminics had only limited success. The convulsive reaction of the antihistamine could be prevented or aborted transitorily in mice treated with 2-methyl-phenyl- α -glyceryl ether (Myanesin), a compound with curare-like action (20).

DISCUSSION

This investigation shows that N,N'-dimethyl-N'-phenyl-N'-(2-thienylmethyl) ethylenediamine hydrochloride (Diatrin) is better tolerated, in all animal species experimented upon, than the other antihistaminics included in this study. Depending upon the route of administration, the average lethal dose of this drug is $1\frac{1}{2}$ –4 times higher than that of five other antihistaminics investigated. A further advantage for this compound is that its comparative tolerance is even greater when determined on the basis of *convulsive dose*, since this dose very likely represents the action of the antihistaminic on the central nervous system.

From a more general pharmacological viewpoint, it is noteworthy that the spread between lethal and convulsive action, as well as the onset and length of

mg/kg. Since fatalities occurred among the rabbits of the W-53 and Pyribenzamine series after the second injection, the daily dose of these two drugs was reduced to 6 mg/kg for the remainder of the experimental period. The time and number of deaths and the convulsion rates in the various groups are summarized in Table 8.

The observations made in this experiment indicate that Diatrin has the lowest toxicity, as shown by the relatively few convulsions and the absence of fatalities in this group.

In a second series of intravenous injection experiments with various antihistamines (Diatrin, Pyribenzamine, Antergan, Neo-antergan, Benadryl), the

TABLE 8

Mortality and convulsion rates in rabbits after repeated intravenous injections of antihistamines—8 mg/kg
(group of 6 rabbits for each drug)

INJECTIONS	2	7	11	17	24	30
Diatrin						
Death	0	0	0	0	0	0
Convls.	0	2	3	4	2	2
Pyribenzamine*						
Death	3	0	0	0	0	0
Convls.	6	2/3†	2/3	2/3	2/3	2/3
W-53*						
Death.	1	0	1	1	0	0
Convls.	6	3/5	3/5	3/4	2/3	2/3
Neo-Antergan.						
Death.	0	0	0	0	0	0
Convls.	6	5	5	5	4	6
Benadryl						
Death.	0	1	1	0	0	0
Convls.	3	6	5/5	4/4	1/4	1/4

* dose reduced to 6 mg/kg after 2nd injection.

† number convulsed/number left alive.

effect of a gradual increase of the dose of antihistaminics during a 6-weeks' period of daily administration was studied. Starting with a relatively low dose, i.e., medium convulsive dose, the amounts finally given to and tolerated by some of the rabbits surviving this type of medication reached at times the LD₅₀, or even LD₁₀₀, of these compounds. This development of tolerance following the daily administration of antihistaminics could not be consistently obtained.

Only the rabbits which died from the effects of the treatment with the various antihistamines showed at autopsy considerable hyperemia of the meningeal vessels, congestion of the liver and occasionally hemorrhages and engorgement of the lungs. A moderate amount of clear serous fluid was found in the peritoneal cavity of 2 rabbits killed by Diatrin and of 1 rabbit succumbing to Neo-antergan. On the other hand, there were no gross pathologic changes in the rabbits killed at the end of the experimental period.

tialities of a drug a number of its antihistaminic and antianaphylactic characteristics should be determined. These findings are in complete agreement with experimental data of Rose, Feinberg, Friedlaender and Feinberg (21), showing that Benadryl, Pyribenzamine, Antergan and Neo-antergan have the same antianaphylactic activity, while their protective activity against multiple lethal doses of histamine might be of a completely different magnitude. Similar discrepancies were noted by Halpern (22) for certain thioldiphenylamine derivatives.

With the present limited knowledge of the mechanism of allergy and anti-allergic action, it is not possible to say which method of testing might have more clinical significance. It seems, however, that the experimental method most frequently used, i.e., the intravenous injection of multiples of the lethal histamine dose, has no bearing on clinical effectiveness. In fact, drugs with widely different activity against intravenous histamine in guinea pig experiments (such as Benadryl, Pyribenzamine, Neo-antergan) are used clinically in the same dosage. It is possible that these differences will be better accounted for when more information is available on the fate of the antihistaminics in the organism. Hypothetically, a variable rate of absorption at different dose levels and methods of administration, for instance, could explain part of these differences.

SUMMARY

N,N'-dimethyl-N'-phenyl-N'-(2-thienylmethyl) ethylenediamine hydrochloride (Diatrin) has been studied for its antihistaminic and toxicological properties. The following findings are reported:

- (1) Doses of 0.1-1.0 γ /cc inhibit the contraction induced by histamine in the isolated intestine and uterus of guinea pigs. The effect of 20-50 γ histamine on the perfused lung is inhibited by 0.5-1.0 mg Diatrin.
- (2) Injected intravenously into dogs, Diatrin interferes with the blood pressure lowering effect of successive histamine injections.
- (3) It protects guinea pigs against lethal histamine asthma in doses of 0.05 mg/kg (subcut.).
- (4) It protects guinea pigs against multiple lethal doses of intravenous histamine. The minimal active dose against 2-5 lethal doses is 0.25-0.50 mg/kg.
- (5) It protects horse-serum sensitized guinea pigs against lethal anaphylactic shock in 0.5 to 1.0 mg/kg doses.
- (6) The therapeutic index of Diatrin is high, particularly by the oral route of administration (300-1200); by subcutaneous administration the index is 80 in anaphylaxis, 800 in histamine asthma.
- (7) From a qualitative standpoint, the pharmacological action of the drug is similar to that of other antihistaminics of the ethylenediamine series.
- (8) The comparative evaluation of toxic, convulsive, and lethal doses in mice, rats, guinea pigs, rabbits, dogs and cats, indicates that the compound is characterized by a high tolerance. Histopathologic studies confirm this finding.
- (9) There is no relationship between the anti-histamine and antianaphylactic

the convulsive state, are not the same for different antihistaminics and routes of administration.

The normal weight gains and the absence of pathological findings in the organs of animals treated for long periods with Diatrin are further indications of the relative non-toxicity of the drug. Pathological changes in the liver and brain were observed only when extremely high, acutely toxic, doses of this—or of the other antihistaminics—were administered repeatedly.

From a qualitative standpoint, the pharmacological action of Diatrin, (toxic symptoms, lowering of blood pressure, increase of respiratory rate) is similar to that noted for other ethylenediamine derivatives (W-53, Pyribenzamine, Antergan).

In relation to the low toxicity of the drug, its therapeutic index (tolerated dose/therapeutic dose) is quite high under different experimental conditions: 40 to 80 in anaphylactic shock; 800 in histamine asthma. (Table 9).

TABLE 9

Therapeutic indices of Diatrin in anaphylaxis and histamine poisoning of guinea-pigs

CONDITION	DIATRIN TREATMENT	TOLERATED DOSE	PROTECTIVE DOSE	THERAPEUTIC INDEX
histamine poisoning				
2 _{LD} intravenous	oral	600	0.5	1200
	subcut.	40	0.25	160
5 _{LD} intravenous	oral	600	2.0	300
	subcut.	40	1.0	40
histamine asthma	oral	600	2.0	300
	subcut.	40	0.05	800
death anaphylaxis	subcut.	40	0.5	80
symptoms anaphylaxis	subcut.	40	1.0	40

The doses required to protect horse-serum sensitized guinea-pigs against anaphylactic shock are about the same (0.5–1.0 mg/kg) as found for the antihistaminic used for comparison (Pyribenzamine). In the experiments on the isolated organs, the activity of Diatrin and of the various drugs compared is approximately the same. On the other hand, larger doses of Diatrin are required to protect against intravenous histamine than is the case with the other antihistaminics, except Benadryl.¹

Thus, the absolute and the comparative figures of protective dosages of the antihistaminic drug vary according to the method used for determination of activity. The variance of activity ratios between different drugs according to the technique used has an obvious practical importance aside from its theoretical interest. It suggests that in order to have sufficient information on the poten-

¹ We have no explanation for the report of P. Viaud (Prod. Pharmaceut. 2: 53, 1947) who mentions this compound as being inactive. Viaud's statement is in disagreement also with Kyredes, Meyer, and Zienty's recent note (J. A. C. S. 69: 2239, 1947) indicating that the preparation has been tested and found active.

NIAARA; A DIGITALIS-LIKE COLOMBIAN ARROW POISON

K. MEZEY¹, C. URIBE-PIEDRAHITA, J. PATAKI AND J. HUERTAS-LOZANO

The César Uribe-Piedrahita Research Laboratories, Bogotá, Colombia, S. A.

Received for publication March 2, 1948

Some Indian tribes of Antioquia, Colombia have used as an arrow poison, the milky exudate of a tree known locally as "pacuru-niaara" according to Piedrahita who reported studies upon this poison in 1920 (1). Ethnologists later reported its continued use (2, 3, 4) and a sample of the prepared arrow poison was examined by Santesson in 1929 (5).

The raw juice of the bark of the "pacuru-niaara", or "poison tree", an *Arto-carpoidea*, genus *Ogcodeia* and probably *Ogcodeia ternstroemiiflora* Midbr. (6) was collected by one of us and was used for the bulk of these experiments. An active principle has been isolated from the juice and some of the studies were performed with it. This principle, "niaarin", is white, amorphous and gives chemical responses characteristic of the cardiac glycosides. The chemical and physical properties are very similar to those of antiarin but it is not identical with it. A later report will deal with the chemistry of "niaarin".

EXPERIMENTAL. 1. *General effects in mammals.* Intramuscular injection of 30 to 60 per cent of the LD₅₀ (table 1) of raw niaara juice in dogs or cats produces repeated vomiting, occasional diarrhea, and marked weakness with ataxia, all developing within 15 to 30 minutes after the injection. Bradycardia and respiratory depression occur early in the course of poisoning.

Later, cardiac rate is more rapid and irregular. Recovery begins within three or four hours and progresses regularly. After an injection of an LD₅₀ death occurs in two to three hours as the result of respiratory depression. The heart contracts feebly and irregularly after respiration fails but artificial respiration does not prevent the cardiac arrest which follows within a minute after respiratory arrest. Niaarin produces the same general effects but is far more potent (table 1) and very irritating.

In addition to manifesting the effects described above, rats also become agitated and develop convulsions, which may not be entirely asphyxial in nature (9). Vomiting is regularly produced in pigeons.

2. *Absorption and excretion.* Subcutaneous tissues absorb niaarin very slowly in dogs; 70 minutes elapse before the onset of systemic effects in dogs during which there is evidence of marked irritation. It is much more rapidly absorbed from intramuscular sites, 20 minutes being sufficient for symptoms to develop. Following intravenous injection the actions of niaarin are exerted almost immediately.

Absorption of the active principle from raw niaara or directly as amorphous niaarin occurs in the gastro-intestinal tract of dogs, but it is incomplete and the dose must be increased 10 to 20 fold. The symptoms of poisoning, vomiting,

¹ Professor of Pharmacology, Javeriana University Medical School, Bogotá.

effectiveness of various antihistaminic preparations. Similarly, the doses protecting against inhaled and injected histamine, or giving protection *in vitro*, do not necessarily have the same relationship in different compounds.

REFERENCES

- (1) FEINBERG, S. M.: J. A. M. A. 132: 702, 1946.
- (2) FRIEDLAENDER, S.: Am. J. Med. 1: 174, 1946.
- (3) FRIEDLAENDER, A. S. AND FRIEDLAENDER, S.: J. Lab. Clin. Med. 31: 1350, 1946.
- (4) LEONARD, F. AND SOLMSEN, U. V.: (J. A. C. S. in press).
- (5) ERCOLI, N., SCHACHTER, R. J., LEONARD, F. AND SOLMSEN, U. V.: Arch. Biochem. 13: 487, 1947.
- (6) ROTH, L. W., RICHARDS, R. K. AND SHEPPERD, I. M.: Fed. Proc. 6: 366, 1947.
- (7) LEE, H. M., DINWIDDIE, W. G. AND CHEN, K. K.: THIS JOURNAL 90: 83, 1947.
- (8) COMBES, F. C.: (To be published).
- (9) TAINTER, M. L., PEDDEN, J. P. AND JAMES, M.: THIS JOURNAL 51: 371, 1934.
- (10) FRIESEN, S. R., BARONOFKY, I. D. AND WANGENSTEEN, O. H.: Proc. Soc. Exp. Biol. and Med. 63: 23, 1946.
- (11) WINTER, C. A.: THIS JOURNAL 90: 224, 1947.
- (12a) HALPERN, B. N.: Arch. Int. Pharmacodyn. 74: 314, 1947.
- (12b) HALPERN, B. N.: Arch. Int. Pharmacodyn. 68: 339, 1942.
- (13) LOEW, E. R., KAISER, M. E. AND MOORE, V.: THIS JOURNAL 83: 120, 1945.
- (14) ROCHA E SILVA, M. AND DRAGSTEDT, C. A.: THIS JOURNAL 73: 405, 1941.
- (15) LAST, M. R. AND LOEW, E. R.: THIS JOURNAL 89: 81, 1947.
- (16) WINDER, C. V. AND THOMAS, R. W.: THIS JOURNAL 91: 1, 1947.
- (17) YONKMAN, F. F., CHESSE, D., MATHIESON, D. AND HANSEN, N.: THIS JOURNAL 87: 256, 1946.
- (18) GRUHZIT, O. M. AND FISKEN, R. A.: THIS JOURNAL 89: 227, 1947.
- (19) MAYER, R. L., HAYS, H. W., BROUSSEAU, D., MATHIESON, D., RENNICK, B. AND YONKMAN, F. F.: J. Lab. & Clin. Med. 31: 749, 1946.
- (20) BERGER, F. M. AND BRADLEY, W.: Brit. J. Pharmacol. 1: 265, 1946.
- (21) ROSE, J. M., FEINBERG, A. R., FRIEDLAENDER, S. AND FEINBERG, S. M.: J. Allergy 18: 149, 1947.
- (22) HALPERN, B. N.: Bull. Soc. Chim. Biol. 29: 309, 1947.

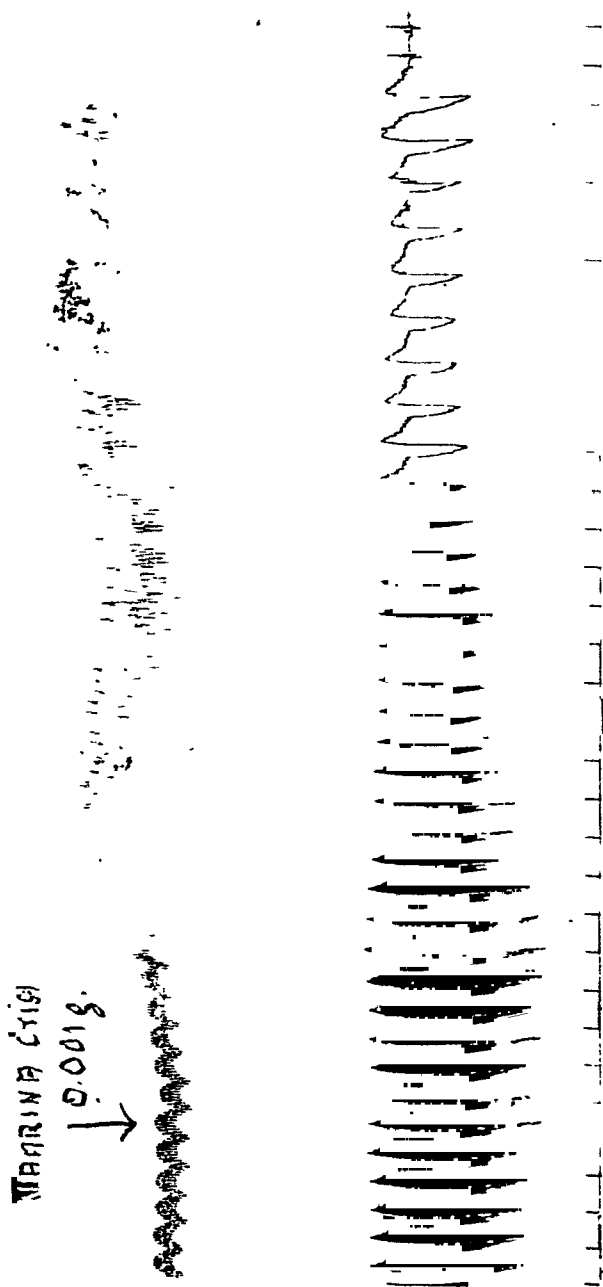


FIG. 1. EFFECT OF TOXIC DOSES OF NIAARIN ON BLOOD PRESSURE AND RESPIRATION OF A CAT
 From above down: carotid artery pressure, respiration and time (6 sec.). Dose intravenously is 0.4 mgm./kgm.

adynamia, and bradycardia are all present but death has never occurred, possibly because of the prompt and persistent vomiting which prevents retention of much of the poison.

When vomiting was prevented by a barbiturate,² oral administration of 10 mgm./kgm. of niaarin (50 times the intravenous LD₅₀) resulted in death within three and one half hours in one dog.

Elimination of niaarin seems to be slow for the symptoms produced by sub-lethal doses persist one or two days. That there is some cumulation is indicated by the following observation. If niaarin is injected intravenously in doses of 0.17 mgm./kgm. 24 hours apart, the first injection produces sub-lethal effects with apparent recovery in eight or ten hours; the second dose causes death.

3. *Effects on the circulatory system.* a) *Blood pressure and respiration.* Fifty dogs and ten cats were anesthetized with 40 mgm./kgm. of a barbiturate,² carotid

TABLE 1

LD₅₀ by various routes of raw niaara juice and amorphous niaarin in several species

SPECIES	ROUTE	NUMBER OF ANIMALS	LD ₅₀	HOURS TO DEATH
Niaara				
Dog.....	i.m.	30	0.05 cc./kgm.	2-3
Cat.....	i.m.	5	0.05 cc./kgm.	2-3
Rabbit.....	i.m.	10	0.08 cc./kgm.	8-14
Rat.....	i.p.	30	1.0-1.1 cc./kgm.	—
Niaarin				
Dog.....	i.v.	8	0.22 mgm./kgm.	10-12 min.
Cat*.....	i.v.	6	0.21 mgm./kgm.	30-35 min.

* By the continuous intravenous injection method described by Mezey (7).

pressure was recorded with a mercury manometer and respiration was recorded from a tracheal cannula. Drugs were injected into the femoral vein.

As little as 0.001-0.002 cc./kgm. of niaara produced a slight rise in blood pressure and simultaneously decreased respiratory rate. Larger doses (0.01 cc./kgm.) produced a rise of 30-50 mm. of Hg in the blood pressure associated with bradycardia and extrasystoles. Still larger doses (0.05-0.1 cc./kgm.) produced a similar effect, followed by a sudden fall of the pressure to zero and death in respiratory arrest. Niaarin in doses of 0.1-0.2 mgm./kgm. is similar in action (figs. 1 and 2).

Neither atropine sulphate, 1.0 mgm./kgm., nor ergotamine tartrate, 0.02 mgm./kgm. altered the action of niaara and niaarin on the blood pressure, but atropine did prevent the bradycardia, while ergotamine prevented respiratory inhibition. Denervation of both carotid sinuses and sections of both vagi and cervical sympathetic trunks had no effect on the blood pressure changes, but

² Sodium phenyl-methyl-butyl barbiturate, "Barbisedan".

sensitive to niaara in dilutions of 1:1000. The effect was typical of the digitalis bodies and 1:500 niaara produced systolic standstill.⁴

4. *Effect on smooth muscle.* The isolated rabbit ileum (Magnus technique) developed considerable increase of tone and amplitude of contraction when exposed to niaara in dilutions of 1:20,000. Niaarin in dilutions of 1:200,000 to 1:300,000 produced the same effect. The effects were readily reversed by washing.

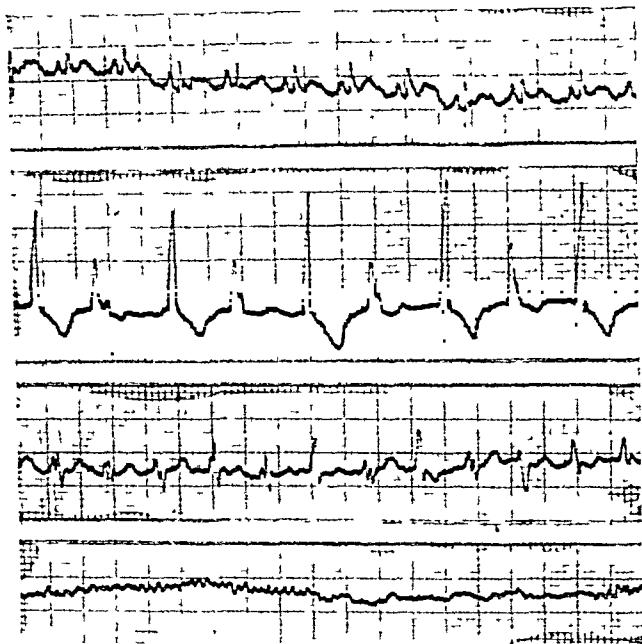


FIG 3 ELECTROCARDIOGRAMS (LEAD II) OF AN ANESTHETIZED CAT RECEIVING 0.135 MG/M OF NIAARIN INTRAVENOUSLY

From above down Control and 1, 3 and 5 minutes after the injection

The tone of isolated uteri was markedly increased by niaara (1:10,000) and niaarin (1:300,000). This effect is also reversed by washing.

Previous treatment of the intestine or uterus with atropine (1:50,000) or with ergotamine (1:100,000) did not inhibit the stimulating effects of either niaara or niaarin. Apparently the action is a direct one on the muscular elements.

5. *Effect on myoneural junction.* Experiments on toads and dogs in which direct and indirect irritability of skeletal muscles was examined failed to reveal any evidence of curare-like action.

⁴ Drs H. Miller and R. Meier of the Department of Pharmacological Investigations of the Ciba Laboratories at Basle very kindly performed the experiments on *R. temporaria*, a species which does not occur in Colombia.

bradycardia was prevented. Apparently, the bradycardia is the result of vagal activity, possibly initiated by the elevated pressure in the carotid sinuses.

b) *Electrocardiogram*.³ The effect of niaarin on the electrocardiogram was studied in one anesthetized cat which received 0.045 mgm./kgm. intravenously, followed one hour later by 0.135 mgm./kgm. Marked slowing, prolongation of the P-R interval and a slight depression of the S-T segment were produced by the small dose. The large dose produced premature ventricular contractions

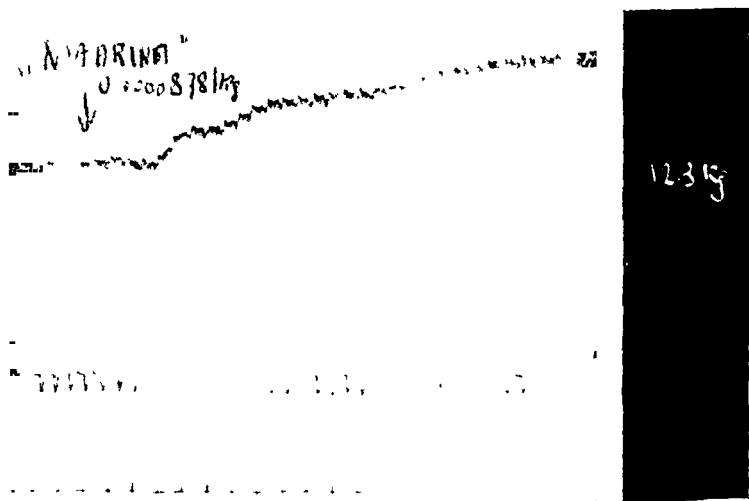


FIG. 2. EFFECT OF 0.088 MG./KG. OF NIAARIN ON THE BLOOD PRESSURE AND RESPIRATION OF A DOG

From above down: carotid artery pressure, respiration and time (6 sec.). The second tracing was made 20 minutes after the first.

depression of the T-wave and five minutes after the injection, ventricular fibrillation (fig. 3).

c) *Isolated mammalian cardiac muscle*. Isolated right ventricular papillary muscle preparations were allowed to fail as described by Cattell and Gold (8). Addition of niaarin in concentrations of 1:1,300,000 produced almost immediate recovery while greater dilutions (1:6,500,000) produced a 300 per cent increase in systolic tension within 26 minutes.

d) *Isolated amphibian heart*. Isolated toad hearts (*Bufo marinus*) were prepared according to the technique of Straub. Niaara decreased the frequency and increased the amplitude of the contraction in the majority of twenty experiments.

Similar isolated frog (*Rana temporaria*) heart preparations were found to be

³ We wish to express our gratitude to Dr. Chenoweth and to Dr. Garb of the Department of Pharmacology, Cornell University Medical College for their help with the electrocardiograms and the experiments on the papillary muscle.

2. NORDENSKIÖLD, E.: Comparative ethnographical studies, **3**: 52 *et seq.*, Göteborg, 1931.
3. KARSTEN, R.: Societas Scientiarum Fennica, **6**: 4, 1933.
4. WASSEN, H.: Journal de la Société des Americanistes, **25**: 103, 1933.
5. SANTESSON, C. G.: Skand. Archiv. f. physiol., **55**: 230, 1929.
6. URIBE, C., AND MEZEY, K.: Universidad Nacional de Colombia (Publicaciones) **3**: 177, 1945.
7. MEZEY, K.: Amer. Jour. Pharmacy, **115**: 326, 1943.
8. CATTELL, MCK., AND GOLD, H.: THIS JOURNAL, **62**: 116, 1938.
9. GOLD, H., MODELL, W., CATTELL, MCK., BENTON, J. G., AND COTLOVE, ELAINE: THIS JOURNAL, **91**: 15, 1947.

6. *Action of niaarin in man.* Three subjects with organically and functionally intact circulatory systems tolerated total intravenous doses of 0.25 to 1.0 mgm. of niaarin very well. Slight slowing of the pulse was observed during the 6 to 8 hours following injection. Diarrhea was also produced.

In 7 cases⁵ of congestive heart failure, niaarin as the sole therapy, was injected intravenously each day in doses of 0.50 to 0.75 mgm. for the first two days and 0.25 mgm. daily for two or three days more. The therapeutic effect was evident in less than 24 hours after initiation of the treatment. Heart rate fell (average fall 40 beats/min.), venous pressure was reduced (average reduction 150 mm. of H₂O), respiratory rate decreased (average decrease 10/min.) circulation time became shorter (average decrease 15 seconds) and considerable diuresis occurred in those patients with marked congestion. Electrocardiograms generally revealed depression of the S-T segment and in three cases, inversion of the T-wave.

On the whole, niaarin compares closely with strophanthin in rapidity and brevity of action. Cumulation is not important when 0.25 mgm. is given as the daily maintenance dose. No side effects were noted in the congestive failure cases.

DISCUSSION. The actions of niaarin are generally typical of the cardiac glycosides, although the gastro-intestinal tract is involved more than usual.

When the source of the poison is confined to the single tree, *Ogcodeia ternstroemiiflora*, no curare-like action is produced by either the raw juice or the purified principle, niaarin. The curarization observed by Santesson with his sample was probably the result of mixing poisons at the source, a custom he describes. Convulsions in rats are now known to be produced by several of the more familiar cardiac glycosides (9).

Although niaarin is an effective therapeutic agent, the necessity for intravenous administration limits its field of usefulness. On the other hand, the actions of niaarin develop with remarkable rapidity which may occasionally be desired.

SUMMARY

1. Niaara, an arrow poison from Colombia, S. A. is the latex of the tree *Ogcodeia ternstroemiiflora* Midbr.

2. An amorphous principle ("niaarin") having chemical and pharmacological properties characteristic of the cardiac glycosides has been isolated from the latex of this tree. Neither the crude nor pure materials have any curariform activity.

3. The intravenous LD₅₀ in cats is 0.21 mgm./kgm. It is poorly absorbed from the gastro-intestinal tract.

4. Seven cases of congestive heart failure in man were successfully treated with niaarin.

REFERENCES

1. URIBE, C.: Apuntaciones sobre la Geografía Médica del Ferrocarril de Urabá, Tesis, Medellín (Colombia), 1920.

⁵ A complete report of these cases will be published later.

Measurements of velocity of hydrolysis were begun 5 minutes after mixing the contents of the flasks. "Initial velocity" is the average velocity during the first 10 minute period, corrected for autohydrolysis, except for the red cell data at the lowest substrate concentrations, when readings were taken every two minutes and the initial velocity estimated by drawing a tangent to the curve through zero. The course of the reactions indicated that

TABLE 1

Velocity of hydrolysis of ACh in varying concentrations by human plasma and erythrocytes and by autohydrolysis. Velocity of hydrolysis of ACh in varying concentrations by human plasma in the presence of fixed concentrations of antimalarial drugs. Three columns at the right of the table give the constants derived from these data, and the last column gives for comparison the dissociation constants (K_i) of the enzyme-inhibitor complexes of red cells from the data of Fig 5. Values not given in the table are red cell velocities: 1.47, 1.03 and 0.68 at substrate concentrations 0.030, 0.015 and 0.007×10^{-2} M respectively.

		SUBSTRATE M $\times 10^2$									V μ l/min.	$K_s \times 10^3$ M		$K_i \times 10^3$
		13.6	7.22	3.74	1.87	.941	.472	.245	.121	.061				
		Initial Velocity μ l CO ₂ /min.												
Plasma No. 1.....		3.33	3.39	3.26	3.09	2.78	2.38	1.94	1.55	0.98	3.4	1.64	—	
Plasma No. 2.....		3.10	3.29	3.08	2.90	2.51	2.15	1.82	1.34	0.94	3.2	1.95	—	
Red Blood Cells.....		0.25	0.42	0.72	1.15	1.63	1.97	2.17	2.17	1.83	2.6	0.208	1.53	
Autohydrolysis.....		1.07	0.61	0.34	0.19	0.12	0.08	0.06	0.06	0.06	—	—	—	
DRUG	M $\times 10^2$										V μ l/min.	$K_i \times 10^3$	$K_i \times 10^3$	RBC $K_i \times 10^3$
Chloroquine*	224	3.34	3.17	2.87	2.31	1.74	1.19	—	—	—	3.6	9.9	4.5	6.1
	1120	2.81	2.31	1.74	1.19	0.69	0.44	—	—	—	3.6	38.0	5.1	
Quinacrine*	20	3.30	3.21	2.87	2.41	1.81	1.34	0.96	—	—	3.5	7.8	0.55	2.4
	100	3.01	2.46	1.88	1.28	0.83	0.54	—	—	—	3.7	35.0	0.49	
Paludrine†	439	2.89	2.88	2.73	2.50	2.00	1.37	1.00	0.50	—	3.0	5.1	27.	340.
	2200	2.26	2.00	1.75	1.25	0.86	0.55	—	—	—	2.4	17.3	28.	
Quinine*	100	3.02	2.75	2.42	1.98	1.60	1.14	0.91	—	—	3.1	7.4	2.8	860.
	1000	1.79	1.32	1.09	0.80	0.63	0.46	—	—	—	2.3	34.0	5.1	
Plasmochin*	30	3.05	2.93	2.68	2.18	1.84	1.27	0.96	—	—	3.2	6.8	0.95	590.
	150	2.73	2.16	1.69	1.17	0.84	0.49	—	—	—	3.4	33.4	0.78	
Quinidine*	4	2.90	2.81	2.46	2.02	1.57	1.21	—	—	—	3.1	8.5	0.096	960.
	20	2.45	1.90	1.43	0.96	0.64	0.49	—	—	—	3.1	39.0	0.088	

* Control: Plasma No. 1.

† Control: Plasma No. 2.

by the time the first reading was made, the drug-enzyme complex was already in equilibrium, and also that no detectable destruction of the drugs occurred during a period of more than two hours. As the determinations are ordinarily made, the drug is in contact with the enzyme for 15 to 20 minutes before measurements are started. In order to investigate the period immediately following addition of inhibitor to reaction mixture, parallel determinations were made with quinaerine and quinidine added to plasma and Ringer's solution (a)

CHOLINESTERASES OF HUMAN ERYTHROCYTES AND PLASMA AND THEIR INHIBITION BY ANTIMALARIAL DRUGS

C. I. WRIGHT AND JEAN C. SABINE

WITH TECHNICAL ASSISTANCE OF ANNE H. WRIGHT

Experimental Biology and Medicine Institute, National Institute of Health

Received for publication March 5, 1948

The work reported here is part of a program (1) undertaken to uncover any relationship between the antimalarial activity or toxicity of chemical compounds and their inhibitory action on cholinesterase. Although no definite relationship was found, enough data were collected to characterize the enzymes both of human plasma and erythrocytes with respect to substrate and inhibitor. Of the six drugs discussed in this paper (see Table 1) all but plasmochin and chloroquine have been reported to inhibit cholinesterase (2, 3, 4, 5). The determinations, however, have been limited as to type of enzyme or range of concentrations of substrate and inhibitor, so that calculations of dissociation constants and relative affinities, comparison of action of different types of cholinesterase, demonstration of reversibility and type of inhibition, competitive or non-competitive, have not been possible.

As shown by Alles and Hawes in 1940 (6), and by many investigators since, the enzyme of red blood cells is qualitatively different from that of plasma, but the data in the literature have not been sufficiently extensive to permit the analysis of its enzyme-substrate kinetics. For this reason a fairly large section of this paper is devoted to the cholinesterase of the erythrocytes in the absence of inhibitors.

METHOD. The inhibition of cholinesterase was determined by measuring the rate of hydrolysis of acetylcholine iodide (*ACh*) using the manometric method introduced by Ammon (7). The general procedure was outlined in a previous publication (1). Plasma and red cell preparations were made by drawing blood from the antecubital vein of a human subject, citrating the blood with 1.6 ml. of 30% sodium citrate per 100 ml. of blood, and centrifuging at once. The plasma was diluted to 5% and the red cells to 1% before addition to the manometer flasks. The amount of diluted red cells or plasma used varied from 0.7 to 0.9 ml., depending on the enzymic activity of the sample. Drugs were added to the enzyme preparation in the main compartment of the flasks and *ACh* was placed in the sidearm.

When relatively large quantities of *ACh* are dissolved in Ringer's solution it is necessary to make appropriate corrections in molarity for an increase in volume. These corrections were determined for the higher concentrations by weighing 1.0 ml. of solution and calculating the actual concentration from the ratio of observed weight to expected weight if no change in volume had occurred. For the lower concentrations the volumes of solutions of *ACh* in 1.0 ml. of solvent were compared directly in a capillary tube with 1.0 ml. of solvent. Fig. 1 shows the volume changes from which the actual molar concentration in the manometer flasks were calculated. The corrections amounts to about 20% at 0.3 *M* and becomes negligible at about 0.005 *M*. All substrate molarities have been corrected for volume effects.

The Ringer-bicarbonate solution of Krebs and Hanseleit (8) was used throughout as suspending medium.

1.61×10^{-3} for plasma 1 and 3.23 and 1.95×10^{-3} for plasma 2. These values fall within the range of values obtained for human serum by other investigators (12, 13, 14).

A theoretical curve has been drawn through the data for the activity of the red cell enzyme in Fig. 2 according to the equation:

$$v = \frac{V(S)}{(S) + K_s + (S)^n/K_2} \quad (\text{Equation 2}) \quad (11: p. 84)$$

where the symbols used in Equation 1 remain the same; n represents the number of molecules of substrate combining with a molecule of enzyme to form the inactive enzyme-substrate complex (ES_n), and K_2 represents the apparent dissociation constant $(ES)(S)^{n-1}/(ES_n)$.

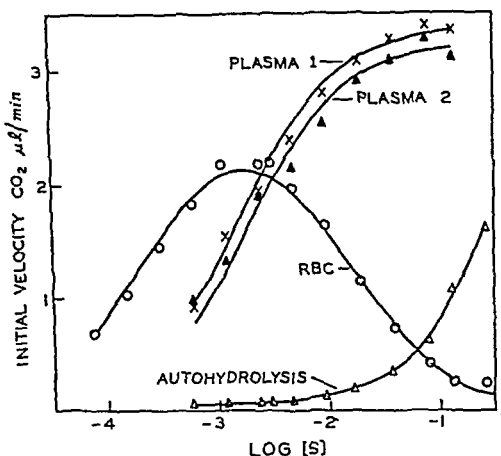


FIG. 2. Velocity of hydrolysis of varying concentrations of *ACh* by human plasma and erythrocytes and by autohydrolysis.

Fig. 3 presents the analysis of the red cell data from Fig. 2 according to the method described by Lineweaver and Burk (15). The constants for the red cell enzyme were determined on the assumption that the cholinesterase has the characteristics of their Case III and the fit obtained supports this assumption. In Graph A the slope represents the reciprocal of V and the intercept represents K_s/V . K_s for the red cell enzyme was found to be 2.1×10^{-4} and V to be 2.6. In Graph B the intercept (at S equal to 1) determines K_2 with a value of 1.5×10^{-2} . The slope is n and equal to 2.0. The value obtained, of 2.0, indicates that one additional molecule of substrate is added to the active ES complex to form the inactive complex.

The optimal substrate concentration was found to be about $2 \times 10^{-3}M$, in close agreement with the values found by earlier workers (6, 16, 17).

When the data for red cell enzyme activity at the five lowest substrate con-

in the usual way (b) 15 minutes after addition of the substrate, and (c) already mixed with the substrate. The substrate concentration (0.02 *M*) was such as to give practically zero-order reactions with time. For both drugs the three determinations gave identical curves from the five minute reading to the termination of the experiment after 1½ hours, indicating that equilibrium is reached within 5 minutes.

Paludrine hydrochloride (SN 12,837) was prepared in this laboratory by Dr. Everette L. May. Quinine (SN 359) and quinacrine (atabrine) (SN 390) were used as hydrochlorides. Chloroquine (SN 7618) was used as a diphosphate; quinidine (SN 1031) as a sulfate; plasmochin¹ (SN 971) as a monohydriodide monohydrate.²

RESULTS. Table 1 and Fig. 2 present the data for the velocity of hydrolysis of *ACh* by human plasma, by erythrocytes and by autohydrolysis in the presence of varying substrate concentrations in the absence of drugs.

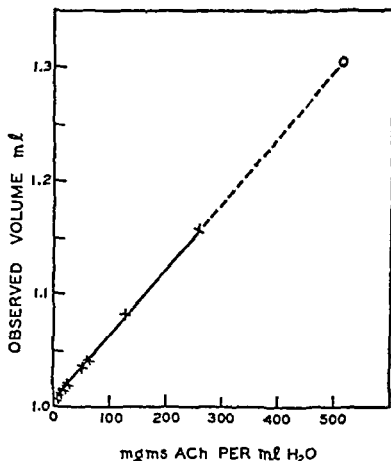


FIG. 1. Volumes of solutions resulting from dissolving the indicated amounts of *ACh* in 1.0ml. H₂O. The value for 512 mgm. is an extrapolation.

The curves drawn through the data for plasma represent the best fit, as determined by the method of least squares (10), using the equation:

$$v = \frac{V(S)}{(S) + K_s} \quad (\text{Equation 1}) \quad (11: \text{p. 39})$$

where v is the initial velocity of hydrolysis at molar substrate concentration (S), V the limiting velocity approached with increasing (S), and K_s the apparent dissociation constant of the enzyme-substrate complex (ES). K_s is equivalent to (S) when v is half of V , at the inflection point of the curve obtained when the data are plotted as in Fig. 2. The values obtained for V and K_s are 3.37 and

¹ Plasmochin monoioidide was furnished by the Winthrop Chemical Company through the kindness of Dr. Lloyd C. Miller.

² Names, spellings and survey numbers used in this paper are from Wiselogle, 1946 (9). Structural formulae are available in the same publication.

ished and with K'_i and K''_i successively increased over K_s , exhibit entirely competitive inhibition. Paludrine successively lowered V' and V'' and increased K'_i and K''_i , so it exhibits a mixture of competitive and non-competitive inhibition. The constants obtained statistically for quinine cannot be expected to yield a simple interpretation of the behavior of this drug since the data cannot be fitted to the theoretical curves.

The validity of K_i for the plasma enzyme-inhibitor complex can be tested by fixing the concentration of *ACh* and varying the inhibitor concentration. In Fig. 4 are shown the data obtained at 0.0374 *M* *ACh* for the six drugs, and also

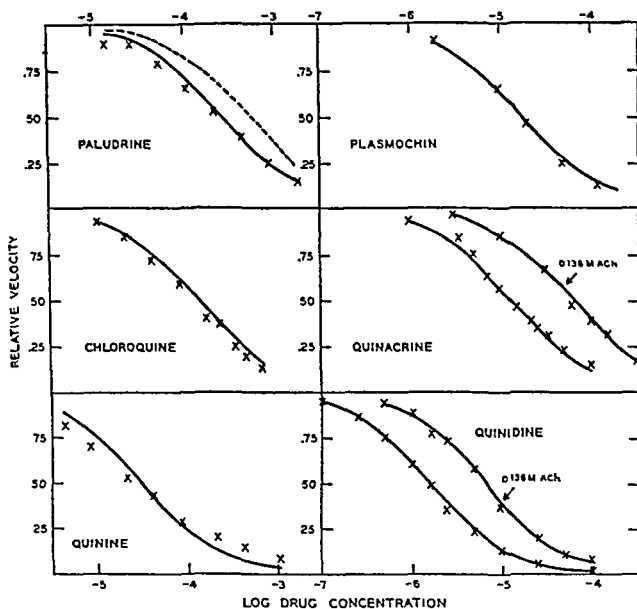


FIG. 4. Relative velocity of hydrolysis of *ACh* by plasma in the presence of varying drug concentrations at substrate concentration 0.0374 *M* for all drugs, and at 0.136 *M* for quinidine and quinacrine.

at 0.136 *M* *ACh* for quinacrine and quinidine. Curves calculated with values for K_s and K_i and V of Table 1 using Equation 3 (which expresses a competitive equilibrium) fit the data for four of the drugs: quinacrine, chloroquine, plasmochin and quinidine. Since paludrine exhibited both competitive and non-competitive inhibition under conditions of varying substrate concentration, the data for this drug would be expected to fit the theoretical competitive curve only after correction for non-competitive inhibition. The theoretical curve has been drawn as a broken line in Fig. 4, and the experimental curve (with an apparent K_i of 1.4×10^{-5}) lies well to the left, as expected. The vertical distance between the two curves at any inhibitor concentration is a measure of the non-competitive

centrations (where $(S)^n/K_2$ is negligible) were fitted directly to Equation 1 by least squares, the values obtained for V and K_s were identical with those obtained by the above analysis.

Table 1 contains the velocities of hydrolysis of *ACh* by plasma at varying substrate concentrations in the presence of fixed concentrations of drugs, and the constants derived from the data. The limiting velocity (V') and the inflection point (K'_s) were determined by the use of Equation 1. K'_s is equivalent to $K_s(1 + \frac{I}{K_i})$, where I is the molar concentration of the inhibitor and K_i is the

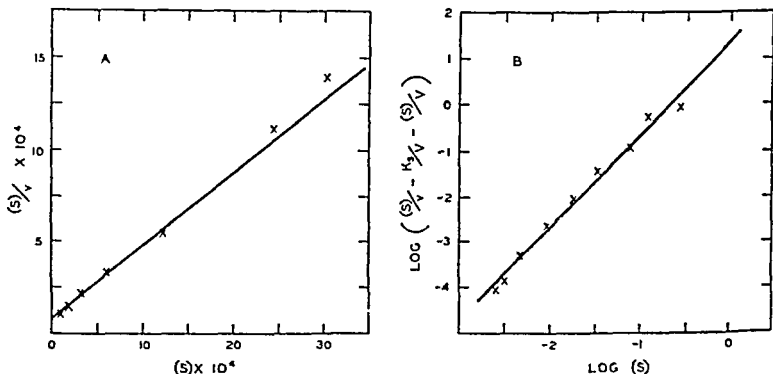


FIG. 3. Evaluation of V , K_s , K_2 and n of the cholinesterase of human erythrocytes from the data of Fig. 2 by the method of Lineweaver and Burk (15, Case III). Graphs A and B correspond to Graphs B and C of their Fig. 3.

dissociation constant of the enzyme-inhibitor complex (EI), so that Equation 1 becomes:

$$v' = \frac{V'(S)}{(S) + K_s \left(1 + \frac{1}{K_i} \right)} \quad (3) \quad (11, \text{p. 46})$$

permitting the calculation of K_i .

When the same limiting velocity is found after the addition of the inhibitor ($V = V'$), any observed inhibition is competitive. When the limiting velocity is lowered by the inhibitor, non-competitive inhibition is present and increasing doses of inhibitor should give successively decreasing limiting velocities ($V > V' > V''$). With competitive inhibition the inflection point of the curve is shifted to the right ($K_s < K'_s < K''_s$); with entirely non-competitive inhibition the value of this constant remains the same as in the absence of inhibitor. When the limiting velocity is decreased and K'_s is greater than K_s , the inhibition is a mixture of these two types.

The data for plasma enzyme and drugs fit Equation 3 well, except for quinine, and the inhibitory action can be interpreted in the manner just outlined. Chloroquine, quinidine, plasmochin and quinacrine, with limiting velocities undimin-

values depend upon the assumptions that all inhibition observed is competitive and that the amount of the inactive complex ES_n is negligible. It is not possible to determine from the data of Fig. 5 whether non-competitive inhibition is present. However, it is possible to estimate the maximum amount of such inhibition which is compatible with the data by finding the greatest variations in V' and K_i which the data will tolerate. Such manipulations have led us to believe that the values for K_i are not likely to deviate from the actual value by a factor greater than 2, and probably are much closer than that.

DISCUSSION: Originally these antimalarial compounds were investigated for their inhibitory action on cholinesterases with the possibility in mind that some relationship might be found between this inhibition and their antimalarial action or acute toxicity. No clearcut relationship of this kind was found. As pointed out earlier (1), this does not preclude the possibility of a close relationship between some toxic symptoms of the drugs, such as gastrointestinal and central nervous system disturbances, or some aspect of their therapeutic action, and their inhibitory action on cholinesterase.

Although no relationship was found between pharmacological action and inhibition of cholinesterase, the data accumulated on the antimalarial compounds investigated here permit an extensive analysis of their activity as inhibitors which may make them useful in investigative work where cholinesterase inhibition is desired. Furthermore the data provide a rather complete characterization of the red cell enzyme-substrate complex which has not been possible heretofore. The widely used competitive inhibitors of cholinesterase, physostigmine and prostigmine, have their limitations in that they are slow to come to equilibrium with the enzyme, and are also destroyed in the reaction mixture (18) (14). This requires arbitrary decisions as to the time of measurements. Four of the six drugs investigated are typical competitive inhibitors; there is no detectable destruction of them in the reaction mixture; and it has been demonstrated experimentally that two of them (quinacrine and quinidine) come to equilibrium very quickly. These two drugs are powerful inhibitors of the cholinesterase found in human plasma, the concentrations required for 50% inhibition being comparable to those reported by Goldstein for physostigmine at equilibrium (14).

Diisopropyl fluorophosphate is an irreversible inhibitor (19) which strongly inhibits the cholinesterase of human plasma at concentrations which do not inhibit the enzyme of red cells. Quinidine offers the alternative of a reversible inhibitor with the same selectivity for the enzyme of human plasma. Quinacrine and chloroquine, on the other hand, are about equally powerful inhibitors of enzymes from both sources.

The ways in which different types of cholinesterases have been classified by various investigators have recently been reviewed (19). These have involved the use of inhibitors or measurements of the relative activity of the enzymes in the presence of different substrates and the effect of high substrate concentrations on activity. As pointed out, none of these methods can be accepted without reservation. An alternative and probably more precise method of classification may be based on the almost ten-fold difference in the acetylcholine-enzyme dis-

inhibition. This confirms the presence of a mixture of the two types of inhibition in the case of paludrine. The quinine data again fail to fit the theoretical curves.

In order to make direct comparisons of the inhibition of cholinesterases of plasma and red blood cells it is necessary to use a relatively low substrate concentration to avoid as far as possible the inhibition of the red cell enzyme by excess substrate. A concentration of $0.003\text{ }M\text{ }ACh$ gives optimal enzymic activity of red cells and also gives approximately equivalent activity of red cells and plasma (cf. Fig. 2). In Fig. 5 are shown the data for inhibition of the enzymes from these two sources by the six antimalarial drugs. It is evident that the

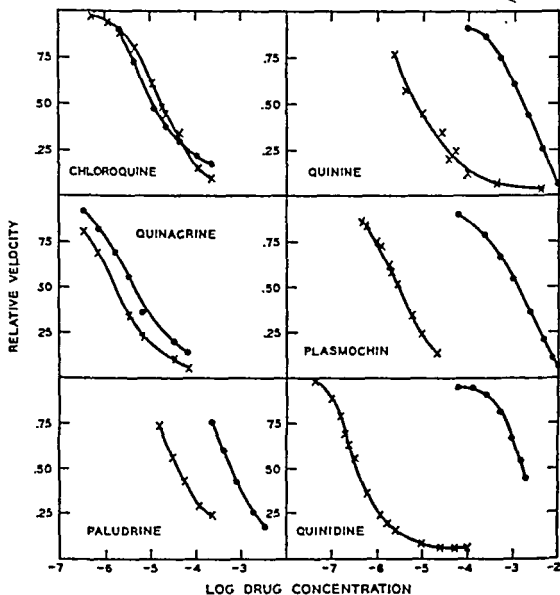


FIG. 5. Relative velocity of hydrolysis of $0.003\text{ }M\text{ }ACh$ by plasma (x) and erythrocytes (●) in the presence of varying drug concentrations.

drugs vary markedly in their relative effectiveness as inhibitors of plasma and red cell enzyme. Chloroquine and quinacrine inhibit the enzyme from both sources about equally, while the other four drugs are much less effective inhibitors of the red cell enzyme.

As Goldstein has shown (14) the ratio I/S is equal to K_i/K_s when the observed relative velocity is 0.5 provided no free enzyme is present. This condition is very nearly met in the experiments with the red cell enzyme since the optimal velocity is about 90% of V . This, therefore, allows the evaluation of K_i for the red cell-inhibitor complex since inhibitor concentration (I) and substrate concentration (S) are experimental quantities and K_s is known from the data of Fig. 2. The values for K_i are shown in the last column of Table 1. These

Dissociation constants of the *EI* complexes are of a different order of magnitude from those for plasma enzyme in the case of paludrine, plasmochin, quinidine (and quinine), all four of these drugs being very much more effective inhibitors of the plasma enzyme than of the red cell enzyme. Quinidine is an excellent selective, competitive inhibitor of plasma cholinesterase as against red cell cholinesterase. The reversibility of its action is in contrast with diisopropyl fluorophosphate.

None of these drugs was destroyed during the reaction. Equilibrium between inhibitor and enzyme was demonstrated in the case of quinacrine and quinidine to be reached within five minutes, whether the drugs were added before, after or coincidentally with the substrate

No clear-cut correlations could be found between the relative inhibiting action on cholinesterase and the antimalarial activity or chemical constitution of these drugs

Dissociation constants K_1 and K_2 were calculated from the data of other investigators (6) (20), and formed a series which indicates a shift of apparent dissociation constant with concentration of sodium and/or potassium ions. The series is made up of data on human erythrocytes and mouse brain, and so gives support to the view that the cholinesterases from these two sources are the same.

REFERENCES

- 1 WRIGHT, C. I. *THIS JOURNAL*, **87**: 109, 1946
- 2 AMMON, R. *Ergebnisse der Enzymforschung*, **4**: 102, 1935
- 3 WAELSCH, H., AND NACHMANSOHN, D. *Proc Soc Exper Biol & Med*, **54**: 336, 1943.
- 4 VINCENT, D., AND BEALJARD, P. *Ann pharm franc*, **3**: 22, 1945
- 5 BLASCHKO, H., CHOI, T. C., AND WAJDA, I. *Brit J Pharmacol & Chemotherapy*, **2**: 116, 1947
- 6 ALLES, G. A., AND HAWES, R. C. *J Biol Chem*, **133**: 375, 1940
- 7 AMMON, R. *Arch f d ges Physiol*, **233**: 486, 1933
- 8 KREBS, H. A., AND HANSELEIT, K. *Ztschr physiol Chem*, **210**: 33, 1932
- 9 WISELOGLE, F. (EDITOR) *A Survey of Antimalarial Drugs* (3v) Ann Arbor, Michigan, J. W. Edwards, 1941-5
- 10 DORN, H. F. *THIS JOURNAL*, **78**: 384, 1943
- 11 HALDANE J. B. S. *Enzymes* London, New York and Toronto, Longmans Green & Co., 1930
- 12 GLICK, D. *Biochem J*, **31**: 521, 1937.
- 13 WRIGHT, C. I., AND SABINE, J. C. *THIS JOURNAL*, **78**: 375, 1943
- 14 GOLDSTEIN, A. *J Genl Physiol*, **27**: 529, 1944
- 15 LINEWEAVER, H., AND BURK, D. *J Am Chem Soc*, **56**: 658, 1934
- 16 NACHMANSOHN, D., AND ROTHENBERG, M. A. *J Biol Chem*, **158**: 653, 1945.
- 17 ZELLER, E. A., AND BISSEGER, A. *Helvet Chim Acta*, **26**: 1619, 1943
- 18 EASSON, L. H., AND STEDMAN, E. *Proc Roy Soc London, Ser B*, **121**: 142, 1936
- 19 BODANSKY, O. *Ann N Y Acad Sci*, **47**: 521, 1946
- 20 MENDEL, B., AND RUDNEY, H. *Biochem J*, **37**: 59, 1943.

sociation constants, since this constant is presumably a characteristic property of an enzyme. However, inspection of Mendel and Rudney's Fig. 1 (20) suggested a shift of K_s of mouse brain to lower values with lowered salt concentration. Because of this suggestion, the data of Mendel and Rudney's Fig. 1 and the data of Alles and Hawes' Fig. 2, Curves III and III-S, were analyzed statistically. The resulting constants are presented in Table 2, along with our own red cell constants. There is a clearly defined shift in the value of K_s from 2.2×10^{-4} at the highest salt concentration to 0.2×10^{-4} at the lowest. There is a similar shift in K_2 based on fewer data. The value of n remained equal to 2 molecules of substrate to one molecule of red cell enzymes.

Direct comparison of K_s of mouse brain and human erythrocyte is not possible because the salt concentrations do not coincide. However, the fact that the values fall into a series when arranged as in Table 2 supports the view that the enzymes from these two sources are the same. At the same time this series

TABLE 2

The effect of sodium and potassium ions on the enzyme substrate dissociation constants (K_s and K_2) of mouse brain and human erythrocytes. The data for these calculations were obtained from (a) Alles and Hawes (b) Mendel and Rudney and (c) this text

ENZYME SOURCE	$\frac{Na^+}{K^+} M$	$K_s \times 10^4 M$	$K_2 \times 10^4 M$	n^*
Mouse Brain (b)	0.185	2.2	—	—
Human RBC (c)	0.149	2.1	1.5	2.0
Human RBC (a)	0.145	1.2	1.3	2.0
Mouse Brain (b)	0.105	1.4	—	—
Mouse Brain (b)	0.065	1.0	—	—
Human RBC (a)	0.066	0.2	0.3	2.3

* n is the number of molecules of substrate required to combine with one molecule of enzyme to form the inactive complex ES_n . (15)

emphasizes the importance of rigid control of experimental conditions. It would seem desirable to make experimental determinations at salt concentrations most likely to exist physiologically.

SUMMARY AND CONCLUSIONS

The inhibition of cholinesterase of human plasma by chloroquine, quinacrine (atabrine), plasmochin, quinidine, quinine and paludrine was measured at varying concentrations of substrate and of inhibitor. Dissociation constants for enzyme-substrate and enzyme-inhibitor complexes were determined. The first four drugs exhibited typical competitive inhibition; paludrine, a combination of competitive and non-competitive; quinine failed to fit the theoretical curves.

The dissociation constants of red cell enzyme-substrate complex (ES) and of the inactive complex (ES_n) were determined to be 2.1×10^{-4} and 1.5×10^{-2} respectively. It was established that in the inactive complex n equals 2.

The inhibition of cholinesterase of erythrocytes by the same drugs in varying concentrations was measured at optimal substrate concentration ($0.003M$).

tered in the food or in the drinking water for the last 12 to 16 days of the experiment. On the last day of study, radioactive iodine was administered intraperitoneally. The animals were sacrificed four hours later. Their thyroids were removed, weighed on a torsion balance and placed separately in 3 cc. of 2% NaOH for digestion.

Three-day old chicks were fed chick starter mash containing the test substances in concentrations of 0.1 to 0.5 per cent for 12 to 14 days. On the last day of each experiment radioactive iodine was administered subcutaneously. The animals were sacrificed four hours later. Their thyroids were removed, weighed and placed in individual bottles containing 3 cc. 2% NaOH.

The radioactive iodine used was the 8-day isotope, I^{131} . It was administered in a dose of 2 microcuries with 2 micrograms of carrier iodine as sodium iodide. The radioactivity was determined as previously described (5). The activity of each digestion mixture was compared with the radioactivity contained in a standard solution prepared at the time of injection of the radioactive iodine.

The chemical compounds studied included thiouracil, 6-propylthiouracil, 6-benzylthiouracil, 2-aminothiazole, 5-aminothiadiazole-2-thiol (TC-68), 3-(phenylaminomethyl)-thiazolidine-2-thione (TC-105) and potassium thiocyanate. These agents were chosen either because of their effectiveness as goitrogens or because of some unusual reaction, e.g., potassium thiocyanate, the goitrogenic effect of which is prevented by the co-administration of iodine.

RESULTS. Observations from two separate groups of experiments on rats carried out independently in two different laboratories are recorded in Table I. Results are tabulated under the following headings: Absolute thyroid weights, thyroid weights per 100 grams of body weight, per cent of administered radioactive iodine collected by thyroids, radioactivity in microcuries per milligram of thyroid tissue, and per cent of control collection, calculated on a basis of microcuries per milligram of thyroid weight.

Comparison of results obtained in the two laboratories indicate quantitative as well as qualitative similarity. Within each group of experiments, thiouracil, TC-68 and TC-105 were observed to be equally goitrogenic especially when calculated on a body weight basis. Likewise, these same agents were found to be qualitatively similar in their effects on the collection of labelled iodine. On a quantitative basis TC-105 appeared to exert a more effective block to the uptake of radioiodine than did the other two agents. Aminothiazole when administered at the same dose level as thiouracil, TC-68 and TC-105 had less goitrogenic activity. It also exerted a much less effective block to the uptake of radioactive iodine.

Potassium thiocyanate administered in the food or drinking water in concentrations just below toxic levels, was considerably less goitrogenic than thiouracil, TC-68 or TC-105. In one experiment goiters produced by this drug were found to concentrate a greater per cent of the administered labelled iodine than did the controls. However, when calculated on the basis of unit mass of thyroid tissue, these goiters collected only 63% as much radioiodine as did the control thyroids. In the second experiment with potassium thiocyanate this agent was found to be much less active as a goitrogen. In this group of animals, though there was a less effective block to the collection of radioactive iodine than that produced by the other agents used, these goitrous thyroids collected less of the

THE EFFECT OF CERTAIN GOITROGENIC DRUGS ON THE ABSORPTION OF RADIOACTIVE IODINE¹ BY THE THYROID GLAND OF RATS AND CHICKS

I. COLLECTION OF RADIOIODINE BY THYROIDS MADE GOITROUS FOLLOWING CHRONIC ADMINISTRATION OF THESE AGENTS

R. W. RAWSON, D. A. MCGINTY, WENDELL PEACOCK, PRISCILLA MERRILL,
MARY WILSON AND HELEN LOCKHART

The Thyroid Clinic of the Massachusetts General Hospital, Boston, Massachusetts; the Research Laboratories, Parke, Davis and Company, Detroit, Michigan; and the Radioactivity Center of the Massachusetts Institute of Technology, Cambridge, Massachusetts

Received for publication March 6, 1948

Tracer studies with radioactive iodine have been used by several investigators in studying certain chemical compounds having goitrogenic activity. Rawson, Tannheimer and Peacock (1) observed in the rat that goiters produced by chronic administration of thiouracil collected less radioiodine than did the thyroids of controls, whereas thyroids made goitrous with potassium thiocyanate trapped a greater proportion of the injected iodine. Franklin, Lerner and Chaikoff (2) obtained similar results with thiouracil in the intact rat but demonstrated by in vitro studies (3) that while surviving sheep thyroids absorb radioiodine from a Ringer-bicarbonate medium containing thiourea, thiouracil or allyl thiourea, little or none of the absorbed iodine was converted into diiodotyrosine or thyroxine as occurred in control experiments when goitrogenic compounds were absent from the medium (4). However, using the same in vitro techniques, Franklin, Chaikoff and Lerner (3) found that thyroid slices failed to collect radioiodine in the presence of potassium thiocyanate.

Thyroids of chicks treated chronically with thiouracil were shown by Larson, Keating, Peacock and Rawson (5) to be resistant to the uptake of injected radioiodine. They found also (6) that a single injection of five milligrams or more of thiouracil produced an effective block to the collection of iodine lasting up to six hours.

We have used the radioactive iodine technic for comparing certain goitrogenic compounds varying considerably in chemical structure and in antithyroid activity as measured by other means (7, 8). This report deals with the collection of radioiodine administered to rats and chicks made goitrous by feeding these chemical agents.

METHODS. Twenty-six to twenty-eight day old rats were maintained on iodine deficient diets for varying periods of time, 12 to 55 days. The goitrogenic compounds were adminis-

¹ The radioiodine used in this investigation was supplied in part by the Isotopes Office, Clinton Laboratories, Monsanto Chemical Co., Knoxville, Tenn., and obtained on allocation by the U. S. Atomic Energy Commission.

trols. TC-105 and TC-68 administered in concentrations just below the toxic levels were found to be only moderately goitrogenic, and in the time interval studied, to exert no block to the collection of radioiodine. Indeed, when the radioiodine collection was calculated on the basis of radioactivity concentrated per milligram of thyroid tissue, chicks treated with these agents were found to collect more iodine than did their controls. Potassium thiocyanate in a concentration of 0.3% in the diet was found to be a very effective goitrogen in the cockerel. The per cent of administered iodine collected by these goiters was considerably greater than that collected by the control thyroids. However, when calculated on the basis of radioactivity per milligram of thyroid tissue, these thyroids concentrated 117% as much iodine as did the controls.

TABLE II

NUMBER OF CHICKS	DRUG PER CENT IN DIET	DAYS ON DRUG	AVERAGE THYROID WT	RADIOIODINE COLLECTION		
				Per cent \pm S.E. mean of adm. radioiodine	Micro-curies per mgm thyroid	Per cent of control uptake per mgm thyroid
			mgm			
9	Controls	—	5.41 \pm 0.81	5.1 \pm 0.51	0.019	100.0
17	0.2% thiouracil	12	16.2 \pm 2.72	2.9 \pm 0.54	0.0036	18.8
10	0.1% propyl TU	12	16.6 \pm 1.33	2.2 \pm 0.34	0.0026	14.0
10	0.1% benzyl TU	12	13.2 \pm 1.10	0.9 \pm 0.45	0.0014	7.3
20	0.5% aminothiazole	12	7.2 \pm 0.54	12.3 \pm 0.79	0.034	181.6
20	0.1% TC-105*	12	10.4 \pm 0.78	12.0 \pm 1.98	0.023	122.1
16	Controls		8.8 \pm 0.56	10.9 \pm 0.84	0.024	100.0
13	KSCN 0.3%	14	19.9 \pm 2.79	27.9 \pm 2.18	0.028	117.0
10	Controls		8.8 \pm 0.95	12.3 \pm 1.87	0.028	100.0
10	TC-68 0.1%†	14	10.6 \pm 3.82	21.9 \pm 4.16	0.041	146.4

* TC-105, 3-(Phenylaminomethyl) thiazolidine 2-thione.

† TC 68, 5 Aminothiadiazole 2-thiol.

DISCUSSION. Radioactive iodine techniques have been used in comparing the effects of seven goitrogenic agents on the natural avidity of thyroid tissue for iodine.

Iodine uptake by the thyroid, estimated from radioactivity measurements, is based on the assumption that injected labelled iodide is not materially diluted by iodide of body fluids and that radioactivity measurements represent proportional amounts of total iodide injected. Such error as does enter is fairly constant from one experiment to another.

In the rat, thiouracil, TC-68 and TC-105 have been observed to have pronounced and almost identical goitrogenic activity. They also inhibit very effectively the avidity of the thyroid for iodine. Although they do not differ greatly in iodine blocking capacity, this inhibiting action is most pronounced with TC-105, followed closely by thiouracil and TC-68. Aminothiazole, which has

administered labelled radioactive iodine than did their controls. Calculated on the basis of radioactivity per unit mass of thyroid tissue, the thyroids of these rats concentrated 46% of that collected by the controls. Since it has been adequately demonstrated that the goitrogenic effect of potassium thiocyanate can be prevented by iodine, it seems quite possible that the differences obtained in these two studies can be explained by the fact that the first group of animals were maintained on an iodine-deficient diet estimated to contain about one-half as much iodine as that contained in the diet used in the second series and for a total of 55 days versus a total of 26 days in the case of the latter group.

Observations made in cockerels fed these drugs for 12 to 14 days are recorded in Table II. The results are tabulated under the following headings: Thyroid

TABLE I

NUMBER OF RATS	DRUG PER CENT IN FOOD	DAYS ON DIET	DAYS ON DRUG	THYROID WEIGHT		RADIOIODINE COLLECTION		
				Mgm \pm S.E.M.	Mg. per 100 gm. rat	Per cent \pm S.E.M. of administered radioiodine	Microcuries per mgm thyroid	Uptake per cent of control per mgm thyroid
16	Control	55		13.7 \pm 0.66	10.3	27.6 \pm 1.98	0.0425	100.0
16	Thiouracil .2%	55	15	44.6 \pm 1.91	32.3	2.1 \pm 0.42	0.00095	2.2
18	TC-68* .2%	55	15	44.3 \pm 2.50	31.5	7.6 \pm 0.52	0.0034	8.1
17	TC-105† .2%	55	15	44.7 \pm 2.11	34.1	0.6 \pm 0.06	0.00028	0.7
16	Aminothiazole .2%	55	15	31.4 \pm 1.86	24.7	19.8 \pm 1.88	0.0126	29.7
16	KSCN .25% (H ₂ O)	55	15	26.8 \pm 2.5	20.9	34.9 \pm 2.58	0.0262	63.3
10	Control	26		10.6 \pm 0.3	9.2	12.3 \pm 0.9	0.0231	100.0
9	Thiouracil .1%	26	16	44.4 \pm 2.7	32.5	4.2 \pm 0.4	0.0019	8.3
9	TC-68 1%	26	16	46.5 \pm 1.7	34.7	5.9 \pm 0.6	0.0025	10.9
9	TC-105 1%	26	16	43.0 \pm 2.1	31.7	2.2 \pm 0.2	0.001	4.4
10	KSCN 25%	26	16	18.1 \pm 1.3	11.5	9.7 \pm 1.3	0.0107	46.4

* TC-68, 5-Aminothiazazole-2-thiol.

† TC-105, 3-(Phenylaminomethyl)-thiazolidine-2 thione

weight, per cent of administered radioiodine collected by the thyroids, uptake per milligram of thyroid tissue in microcuries of radioactivity, and per cent of control uptake on the basis of microcuries per milligram of thyroid tissue.

It was observed that thiouracil when administered in a concentration of 0.2% in the diet had a goitrogenic effect about equal to that of propylthiouracil and benzylthiouracil when administered in concentrations of 0.1% in the diet. Goiters produced by these agents were found to have a markedly decreased avidity for radioactive iodine. From these observations benzylthiouracil appears to be more effective in blocking the collection of iodine than do the other agents. Aminothiazole when administered in a dose of 0.5% in the diet had very little goitrogenic activity. However, thyroids of chicks fed this agent were observed to concentrate 181% as much radioiodine per milligram of tissue as did the con-

thiouracil, 5-aminothiadiazole-2-thiol (TC-68), 3-(phenylaminomethyl)-thiazolidine-2-thione (TC-105), aminothiazole and potassium thiocyanate.

In the rat, thiouracil, TC-68 and TC-105, all highly goitrogenic over the period of treatment, produced effective blocks to the collection of iodine. Aminothiazole was less effective in its blocking action. Goiters produced by KSCN absorb approximately the same amount of injected radioiodine as do thyroids of untreated controls but somewhat less per milligram of thyroid tissue.

In the chick, goiters due to thiouracil and its analogs, benzylthiouracil and propylthiouracil, collect much less iodine than controls. Enlarged thyroids from chronic treatment by aminothiazole, TC-68, TC-105 and KSCN had a greater than normal avidity for radioiodine.

REFERENCES

1. RAWSON, R. W., TANNHEIMER, J. F., AND PEACOCK, W.: *Endocrinol.*, **34**: 245, 1944.
2. FRANKLIN, A. L., LERNER, S. R., AND CHAIKOFF, I. L.: *Endocrinol.*, **34**: 265, 1944.
3. FRANKLIN, A. L., CHAIKOFF, I. L., AND LERNER, S. R.: *J. Biol. Chem.*, **153**: 151, 1944.
4. MORTON, M. E., AND CHAIKOFF, I. L.: *J. Biol. Chem.*, **147**: 1, 1943.
5. LARSON, R. A., KEATING, F. R. JR., PEACOCK, W., AND RAWSON, R. W.: *Endocrinol.*, **36**: 149, 1945.
6. LARSON, R. A., KEATING, F. R., JR., PEACOCK, W., AND RAWSON, R. W.: *Endocrinol.*, **36**: 160, 1945.
7. ASTWOOD, E. B., BISSELL, A., AND HUGHES, A. M.: *Endocrinol.*, **37**: 456, 1945.
8. MCGINTY, D. A., AND BYWATER, W. G.: *THIS JOURNAL*, **84**: 342, 1945; **85**: 129, 1945.
9. WOLFF, J., CHAIKOFF, I. L., TAUROG, A., AND RUBIN, L.: *Endocrinol.*, **39**: 140, 1946.
10. RANDALL, R. A., AND RAWSON, R. W. (unpublished).
11. VANDERLAAN, J. E., AND VANDERLAAN, W. P.: *Endocrinol.*, **40**: 403, 1947.

less goitrogenic activity, has likewise less inhibiting effect on the thyroid's avidity for iodine.

In contrast to the action of thiouracil, TC-68, TC-105 and aminothiazole, potassium thiocyanate is weakly goitrogenic in the rat. Likewise uptake of iodine per unit thyroid weight is only slightly less than that of untreated controls. These results are in good conformity with those obtained earlier by Rawson, Tannheimer and Peacock (1) when their observations are calculated on the basis of unit of radioiodine uptake per unit mass of thyroid tissue.

Collection of radioiodine by potassium thiocyanate-treated rats is quite different from that observed by Franklin, Chaikoff, and Lerner (3) who observed that thyroid slices *in vitro* fail to absorb radioiodine if this agent is contained in the nutrient medium in .01 M concentration. In a subsequent *in vivo* study, Wolff, Chaikoff, Taurog and Rubin (9) observed that the iodine-concentrating capacity is depressed in rats treated with KSCN, provided high concentrations of the compound are present in the circulation at the time of administering radioiodine. They found also that the enlarged thyroid of the KSCN-treated rat had a greater than normal capacity for iodine provided KSCN could no longer be demonstrated in the plasma. Randall and Rawson (10) have found a diminished capacity of rats' thyroids for collecting radioiodine, one to six hours following subcutaneous injection of 10 milligrams of KSCN. This block was no longer demonstrable after 12 hours.

In chicks quite different results were obtained with some of the drugs studied. Goiters produced by thiouracil, propylthiouracil and benzylthiouracil handle iodine as do goiters produced by thiouracil in the rat. The iodine-blocking action of these compounds was greatest with benzylthiouracil followed by propylthiouracil and thiouracil. Goiters produced in the chick with TC-68 and TC-105, both of which effectively inhibit iodine collection in the rat, have a greater than normal avidity for iodine.

These observations might lead one to believe that these goitrogenic agents in this species act through different mechanisms than prevail in the rat or that they undergo some chemical transformation in the chick. Aminothiazole which was observed to have very little goitrogenic activity in the cockerel appears to augment by a considerable degree the chick thyroid's avidity for iodine. This observation is also different from that observed in the rat. In the chick, KSCN was observed to be a very effective goitrogen. However, the goiters which resulted from administering this agent manifested no block to the collection of iodine. Indeed, these thyroids collected 117% as much iodine per unit mass of tissue as that collected by the controls. Apparently the inhibitory action of KSCN on thyroxine synthesis (9) is more pronounced during chronic administration of this drug than its inhibitory effect on iodide absorption (11).

CONCLUSIONS

The capacity of the thyroid gland to collect radioiodine has been studied following chronic treatment of rats and chicks with thiouracil, benzylthiouracil, propyl-

(phenylaminomethyl)-thiazolidine-2-thione (TC-105), 2-aminothiazole and potassium thiocyanate. Selection of these agents was based in part on considerable laboratory experience involving various other techniques and in part on the basis of a wide range of goitrogenic potency as judged from chronic feeding tests (5).

METHODS. *Rats.* Female rats of the Sprague-Dawley strain were used throughout the study. These rats at the beginning of each study were between 26 and 28 days old. In order to create a greater absorptive capacity of the thyroid gland for injected iodine, the animals were maintained on a low iodine diet for 4 to 8 weeks prior to treatment with the goitrogenic drug. On the day of each experiment, rats were divided into groups of 8 to 11 animals and injected subcutaneously with varying amounts of the test compounds suspended in 1 cc. of a 10 per cent solution of acacia. At intervals of one hour, eight hours, twenty-four hours and in some cases, forty-eight hours, groups were injected intraperitoneally with 1 cc. of a solution of sodium iodide containing 2 microcuries of I^{131} (the 8-day half-life radioactive isotope of iodine), and 2 micrograms equivalent of iodine as carrier. Uniformly, animals were sacrificed four hours after injecting the radioiodine. Thyroids were removed, weighed and placed in vials containing 3 cc. of a 2 per cent solution of sodium hydroxide. The per cent uptake of administered radioiodine by the thyroids was calculated from radioactivity measurements of aliquots of digested thyroid tissue and of appropriate volumes of the original solution injected. In each experiment the per cent of radioiodine collected by experimental animals was compared to that collected by the untreated controls and was expressed as per cent of the control uptake of radioiodine.

Chicks. Day-old sex linked cockerels were placed in brooders and maintained on chick starter mash for three days. Beginning on the fourth day each chick received by subcutaneous injection, 0.5 unit (Junkmann-Schoeller) of thyrotropic hormone for three successive days. On the following day, chicks were divided into groups of 10 to 20 animals and injected subcutaneously with varying amounts of the goitrogenic drugs suspended in 1 cc. of 10 per cent acacia solution.

In the first series of experiments the minimal dose necessary to produce a maximum block to the collection of iodine was determined. The "maximum block" was arbitrarily defined as 10 per cent or less of the control uptake. This minimal dose was determined by administering acacia suspensions of the test drugs in varying amounts by subcutaneous injection on the fourth day after beginning treatment with thyroid stimulating hormone. One hour later all animals received one-half cubic centimeter of a solution containing 2 micrograms of iodine as sodium iodide and 2 microcuries of radioactive iodine, I^{131} . All animals were sacrificed 4 hours after administration of the radioactive iodine. Their thyroids were removed by block dissection and placed in 3 cc. of 2 per cent sodium hydroxide solution for digestion. The radioactive iodine collected by each thyroid was calculated by determining the radioactivity in aliquots of each digest and comparing it with the activity contained in a standard taken from the solution of radioactive iodine prepared for injection. The per cent of the control uptake was determined by dividing the per cent of radioiodine collected by the test animals' thyroids by that per cent of iodine collected by the control chicks' thyroids.

In the second series of experiments the duration of block to the collection of iodine by the minimal effective dose was determined. Chicks used in this study were also treated with 0.5 Junkmann-Schoeller unit thyroid stimulating hormone for three successive days. On the fourth day all test animals received what had been previously determined to be the minimal effective thyroid-inhibiting dose of drug in a 1 cc. suspension of 10 per cent acacia solution by the subcutaneous route. Groups of 10 to 20 chicks received radioactive iodine as described under the first series of experiments at one, six, twelve and twenty-four hours after injecting the drugs. Controls received radioactive iodine at one and 24 hours after the assay animals had been injected with the test agent. All animals were sacrificed four hours

THE EFFECT OF CERTAIN GOITROGENIC DRUGS ON THE ABSORPTION OF RADIOACTIVE IODINE¹ BY THE THYROID GLAND

II. COLLECTION OF RADIOIODINE BY THYROIDS OF RATS AND CHICKS FOLLOWING A SINGLE INJECTION OF THESE AGENTS

D. A. MCGINTY, R. W. RAWSON, R. G. FLUHARTY, MARY WILSON,
CHARLOTTE RIDDELL AND HILDA YEE

The Research Laboratories of Parke, Davis and Company, Detroit, Michigan, The Thyroid Clinic of the Massachusetts General Hospital, Boston, Massachusetts, and the Radioactivity Center of the Massachusetts Institute of Technology, Cambridge, Massachusetts

Received for publication March 6, 1948

With radioiodine tracer techniques, it has been demonstrated by Rawson, Tannheimer and Peacock (1), Franklin, Lerner and Chaikoff (2) and Larson, Keating, Peacock, and Rawson (3), that the oral administration of thiouracil to rats and chicks for periods of several days results in a marked reduction in the natural capacity of thyroid tissue to concentrate iodine. Recovery from this block to the iodine-concentrating mechanism varied between the rat and chick experiments but apparently was complete in from 24 to 48 hours. In another study, Larson, Keating, Peacock and Rawson (4) demonstrated that a single subcutaneous injection of 10 mgm. of thiouracil markedly inhibited the collection of radioiodine by the chick thyroid. The effect of this dose of thiouracil persisted for 12 hours but was largely dissipated in 48 hours. These investigators also demonstrated that the degree of inhibition to radioiodine collection by the thyroid, produced by administering varying doses of thiouracil, diminished with decreasing amounts of thiouracil and varied as a linear function of the logarithm of the dose. These experiments suggested that this technic might be employed in quantitating the antithyroid properties of other goitrogens both as to their immediate effectiveness and as to their duration of action.

In the experiments being reported, iodine collection by the thyroid was determined in rats and cockerels following single subcutaneous injections of suspensions of several goitrogenic drugs administered in varying amounts. The duration of block to the collection of iodine produced by the injection of these agents was determined by administering tracer doses of radioiodine in the rat at one, eight, twenty-four, and in some cases, forty-eight hours after injecting the test agent; in the chick at one, six, twelve and twenty-four hours after administering the drug.

With this method of study, the anti-thyroid activity of several compounds was quantitated. The compounds used were thiouracil, 6-n-propylthiouracil, 6-benzylthiouracil, 6-methylthiouracil, 5-aminothiadiazole-2-thiol (TC-68), 3-

¹ The radioiodine used in this investigation was supplied in part by the Isotope Office, Clinton Laboratories, Monsanto Chemical Company, Knoxville, Tennessee, and obtained on allocation by the U. S. Atomic Energy Commission.

the 0.25 mgm. dose level. The duration of action of TC-68 appeared to be definitely less than that of thiouracil.

TC-105 in chronic rat experiments was estimated to have a goitrogenic activity of approximately one and one-half times that of thiouracil. However, its acute effect on the collection of radioiodine by the rat's thyroid was considerably less than that exerted by thiouracil. One milligram was required to produce a block equivalent to that caused by 0.25 mgm. thiouracil at one hour (table 3). Figure 1 shows graphically the effect of diminishing doses of the various antithyroid

TABLE 1

NO OF RATS	DRUG	DOSE	HOURS AFTER DRUG, I ¹³¹ ADMINISTERED	RATIO PER CENT I ¹³¹ COLLECTION, EXPERIMENTAL TO CONTROLS, S.E.M.	PER CENT OF CONTROL UPTAKE
		mgm			
8	Thiouracil	5.0	1	0.2 ± 0.3 / 5.6 ± .5	3.6
8	"	5.0	8	0.3 ± 0.2 / 5.6 ± .5	5.4
8	"	5.0	24	2.1 ± .5 / 5.6 ± .5	37.5
8	"	1.0	1	0.7 ± 0.7 / 11.0 ± .2	6.4
10	"	0.5	1	2.1 ± .3 / 30.5 ± 1.7	6.9
9	"	0.5	8	2.2 ± .2 / 30.5 ± 1.7	7.2
9	"	0.25	1	6.2 ± .7 / 24.7 ± 3.3	25.1
9	"	0.25	8	6.5 ± .8 / 24.7 ± 3.3	26.3
10	6-n-Propyl-Thiouracil	0.5	1	1.1 ± .08 / 12.3 ± .9	8.9
11	"	0.5	8	1.0 ± .07 / 12.3 ± .9	8.1
9	"	0.25	1	1.9 ± .2 / 24.7 ± 3.3	7.7
9	"	0.25	8	2.1 ± .2 / 24.7 ± 3.3	8.5
9	"	0.12	1	1.1 ± .08 / 10.8 ± .9	10.2
9	"	0.12	8	0.8 ± .03 / 10.8 ± .9	7.4
9	"	0.12	24	2.1 ± .2 / 10.8 ± .9	19.4
10	"	0.06	1	1.2 ± .04 / 10.8 ± .9	11.1
10	"	0.06	8	1.4 ± .13 / 10.8 ± .9	12.9
10	"	0.06	24	4.3 ± .9 / 10.8 ± .9	39.8
10	"	0.06	48	5.7 ± .7 / 4.2 ± .4	136.0
10	"	0.03	1	3.0 ± .1 / 10.8 ± .9	27.7
10	"	0.03	8	4.5 ± .1 / 10.8 ± .9	41.6

compounds studied on the collection of radioiodine administered one hour after injecting these agents.

Chicks. In general, all of the agents tested exerted some inhibitory effect to the collection of iodine by chick thyroids. In most instances the minimal effective dose was considerably greater than that observed in rats (see table 4 and figure 2).

Thiouracil and propylthiouracil were equally effective, the minimal effective dose of each being 5.0 mgm. in contrast to 0.5 and 0.06 mgm. respectively in the rat. Benzylthiouracil was observed to be the most effective agent in blocking the

after receiving the radioiodine. The thyroids of these chicks were removed and digested in 2 per cent sodium hydroxide solution. The per cent of injected radioiodine collected by the thyroid of each chick was determined as described above. The per cent of the control uptake was also calculated as described above. The one-hour, six-hour and 12-hour groups were compared to the controls killed with the one-hour group. The 24-hour groups were compared to the controls sacrificed at the same time.

RESULTS. Rats. In tables 1 to 3 are shown the number of rats per group, the compound injected, the amount administered, the time interval between injection of the compound and administration of radioiodine, the ratio of percentage uptake of radioiodine by treated rats to the percentage uptake of parallel control groups not treated with goitrogen but given radioiodine on the same day. The latter calculation, also expressed on a percentage basis in the last column, gave good correlation between experimental data obtained on different groups, which on different experimental dates showed wide variations in avidity of their thyroids for iodine.

While the data for thiouracil are limited, it may be observed from table 1 that 5.0, 1.0, and 0.5 mgm. doses of this compound exerted maximal inhibitory action on radioiodine uptake one hour following injection while a dose of 0.25 mgm. was only partially inhibitory. Doses of 5.0 mgm. and as low as 0.5 remained effective for eight hours but even the larger amount was relatively ineffective after 24 hours. These results are in good agreement with those obtained by Larson et al. on chicks (3).

Propylthiouracil (table 1) and benzylthiouracil (table 2) were considerably more active than thiouracil. In the case of each drug, doses of 0.06 mgm. caused nearly maximal block which lasted for eight hours and was evident to a considerable degree at the end of 24 hours. Doses of 0.03 mgm. produced at one hour approximately a 70 per cent block to the collection of labeled iodine. At 8 hours, this block amounted to 50 per cent of the control collection. Inhibition of iodine uptake following 0.015 mgm. doses of benzyl thiouracil was transient, disappearing completely after 8 hours.

Quantitatively, the results gotten with methylthiouracil were similar to those obtained with thiouracil, 0.5 mgm. being the least amount of this which at one hour produced more than a 90 per cent block to radioiodine absorption. At the next lower dose level (.25 mgm.), methylthiouracil allowed absorption of 15 per cent of injected radioiodine at one hour, whereas 25 per cent absorption occurred in the case of thiouracil. This difference, while mentioned, is not considered a reliable index of dissimilarity between these two compounds. The blocking action of 0.5 mgm. methylthiouracil was largely dissipated in 24 hours, whereas a 1.0 mgm. dose exerted a definite effect at this time interval.

TC-68, a compound which in chronic feeding studies has been demonstrated to be more goitrogenic than thiouracil (5) was found to be less effective in blocking the collection of radioiodine following single injections of the drug. Doses of 1.0 and 0.5 mgm. produced a less effective block than did corresponding amounts of thiouracil although differences between the two compounds were less marked at

effect. Inhibition of iodide absorption by rat's thyroids was observed by Vanderlaan and Vanderlaan following injection of as little as 0.1 mgm. KSCN (6). Aminothiazole produced a maximum effect when administered in a dose of 10 mgm.

The duration of block to the collection of iodine produced by the previously established minimal effective doses of these test agents is recorded in table 5 and shown graphically in figure 3. In general, the block to iodine collection was maximum at one hour and had begun to show a decreasing effect at 6 hours in the case of all agents tested. Escape from the block was complete 24 hours after

TABLE 3

NO. OF RATS	DRUG	DOSE	HOURS AFTER DRUG; I ¹³¹ ADMINISTERED	RATIO PER CENT I ¹³¹ COLLECTION, EXPERIMENTAL TO CONTROLS, S.E.M.	PER CENT OF CONTROL UPTAKE
		mgm.			
8	TC-68*	5.0	1	0.3 ± .02 / 5.6 ± .5	5.4
8	"	5.0	8	1.3 ± .5 / 5.6 ± .5	23.2
8	"	5.0	24	4.7 ± .6 / 5.6 ± .5	83.9
8	"	1.0	1	2.0 ± .09 / 11.0 ± .2	18.2
11	"	0.5	1	5.6 ± .5 / 30.5 ± 1.7	18.3
10	"	0.5	8	8.9 ± 1.1 / 30.5 ± 1.7	29.2
9	"	0.25	1	6.6 ± .6 / 24.7 ± 3.3	26.7
9	"	0.25	8	15.2 ± 1.9 / 24.7 ± 3.3	61.5
8	TC-105†	5.0	1	3.3 ± .7 / 38.4 ± 2.5	8.6
8	"	5.0	8	3.4 ± .3 / 38.4 ± 2.5	8.9
8	"	5.0	24	14.9 ± 1.7 / 38.4 ± 2.5	38.8
8	"	1.0	1	2.5 ± .2 / 11.0 ± .2	22.7
11	"	0.5	1	9.6 ± 1.3 / 30.5 ± 1.7	31.4
10	"	0.5	8	8.6 ± .2 / 30.5 ± 1.7	28.2
9	"	0.25	1	16.0 ± 2.0 / 24.7 ± 3.3	64.8

* TC-68, 5-Aminothiadiazoole-2-thiol.

† TC-105, 3-(Phenylaminomethyl)-thiazolidine-2-thione.

administering either 5 mgm. of thiouracil, 5 mgm. of propylthiouracil or 0.5 mgm. of benzylthiouracil. Twenty-four hours after administering either 10 mgm. of TC-105, 5 mgm. of TC-68, 10 mgm. of aminothiazole or 10 mgm. of potassium thiocyanate, there still existed a partial block to the collection of iodine. It is interesting that aminothiazole, a relatively weak antithyroid compound in a dose of 10 mgm., exerted in the neighborhood of a 50 percent block to the collection of iodine at the end of 24 hours.

DISCUSSION. One antithyroid effect of certain goitrogens has been quantitatively compared by determining the capacity of these agents in varying doses to inhibit the collection of iodine by the thyroids of rats and cockerels. In the rat, it has been observed that benzylthiouracil and propylthiouracil have an anti-

collection of iodine by the chick thyroid. The minimal effective dose of this agent in the chick was 0.5 mgm., one-tenth as great as that of thiouracil and propylthiouracil. The minimal effective dose of TC-68 was 5.0 mgm., the same dose as that which produced the maximum effect in the rat. TC-105 in a dose of

TABLE 2

NO. OF RATS	DRUG	DOSE	HOURS AFTER DRUG; I ¹³¹ ADMINISTERED	RATIO PER CENT I ¹³¹ COLLECTION, EXPERIMENTAL TO CONTROLS, S.E.M.	PER CENT OF CONTROL UPTAKE
		mgm.			
10	6-Benzyl Thiouracil	0.25	24	0.9 ± .12 / 5.7 ± .4	15.8
10	"	0.12	1	0.4 ± .04 / 5.7 ± .4	7.0
9	"	0.12	8	0.5 ± .06 / 5.7 ± .4	8.8
10	"	0.12	24	0.9 ± .06 / 5.7 ± .4	15.8
10	"	0.12	48	1.8 ± .7 / 4.2 ± .4	114.0
10	"	0.06	1	0.7 ± .05 / 5.7 ± .4	12.3
11	"	0.06	8	0.5 ± .03 / 5.7 ± .4	8.8
10	"	0.06	24	2.1 ± .3 / 5.7 ± .4	36.9
10	"	0.06	48	5.4 ± .5 / 4.2 ± .4	129.0
10	"	0.03	1	1.6 ± .2 / 5.7 ± .4	28.1
10	"	0.03	8	2.0 ± .2 / 4.2 ± .4	47.7
10	"	0.03	24	3.0 ± .3 / 4.2 ± .4	71.5
10	"	0.03	48	5.2 ± .7 / 4.2 ± .4	124.0
10	"	0.015	1	0.9 ± .09 / 4.2 ± .4	21.4
10	"	0.015	8	3.8 ± .4 / 4.2 ± .4	90.5
10	"	0.015	24	4.1 ± .3 / 4.2 ± .4	97.6
9	6-Methyl Thiouracil	1.0	1	1.1 ± .1 / 19.1 ± 1.4	5.8
9	"	1.0	8	1.8 ± .3 / 19.1 ± 1.4	9.4
9	"	1.0	24	6.7 ± .8 / 12.4 ± 1.6	54.0
9	"	0.5	1	1.4 ± .1 / 19.1 ± 1.4	7.3
9	"	0.5	8	2.5 ± .4 / 19.1 ± 1.4	13.1
9	"	0.5	24	10.6 ± 1.1 / 12.4 ± 1.6	85.4
9	"	0.25	1	3.7 ± .4 / 25.4 ± 3.2	14.6
9	"	0.25	8	5.2 ± .5 / 25.4 ± 3.2	20.5
9	"	0.25	24	17.4 ± .3 / 19.1 ± 2.6	91.1
9	"	0.12	1	6.6 ± 1.1 / 25.4 ± 3.2	26.0
9	"	0.12	8	6.8 ± .8 / 25.4 ± 3.2	26.7
9	"	0.12	24	19.3 ± 1.7 / 19.1 ± 2.6	101.0
9	"	0.06	1	13.2 ± 1.4 / 25.4 ± 3.2	51.9
9	"	0.06	8	10.3 ± 1.2 / 25.4 ± 3.2	40.6
9	"	0.06	24	21.5 ± 2.3 / 19.1 ± 2.6	113.0

10 mgm., failed to produce the block which we had arbitrarily defined as the maximum block. However, in a subsequent study of the duration of block, a 10 mgm. dose of this agent did produce an effective block (see table 5). Potassium thiocyanate produced a maximum block to the collection of iodine when given in a dose of 25.0 mgm. The dose of 10 mgm. also produced a near maximum

TABLE 5

DRUG AND DOSE	HOURS RaI ADMINISTERED AFTER INJECTION OF THE DRUG	% OF ADMINISTERED I^{131} COLLECTED BY THE THYROID S.E.M.	% OF CONTROL COLLECTION
Thiouracil, 5 mgm.	Control	13.7 \pm 2.0	
	1	0.2 \pm 0.03	1.6
	6	1.2 \pm 0.9	8.6
	12	3.6 \pm 2.0	26.6
	24	2.7 \pm 1.3	156.0
	Control (24 hrs.)	1.8 \pm 1.0	
Propyl thiouracil, 5 mgm.	Control	12.3 \pm 1.1	
	1	0.4 \pm 0.08	3.1
	6	2.1 \pm 0.5	17.3
	12	10.2 \pm 1.7	82.9
	24	8.0 \pm 0.7	123.0
	Control (24 hrs.)	6.5 \pm 0.6	
Benzyl thiouracil, 0.5 mgm.	Control	12.3 \pm 1.1	
	1	0.6 \pm 0.08	5.1
	6	1.4 \pm 0.3	11.5
	12	6.3 \pm 1.5	50.8
	24	7.6 \pm 0.5	116.0
	Control (24 hrs.)	6.5 \pm 0.6	
TC-105, 10 mgm.	Control	34.4 \pm 3.1	
	1	3.0 \pm 0.7	8.6
	6	12.4 \pm 1.7	36.1
	12	17.2 \pm 2.7	49.8
	24	14.3 \pm 4.0	73.5
	Control (24 hrs.)	19.4 \pm 3.4	
TC-68, 5 mgm.	Control	15.0 \pm 1.6	
	1	0.2 \pm 0.04	1.1
	6	0.9 \pm 0.3	5.9
	12	15.7 \pm 1.2	104.0
	24	10.7 \pm 2.1	79.9
	Control (24 hrs.)	13.6 \pm 2.0	
Aminothiazole, 10 mgm.	Control	23.7 \pm 1.6	
	1	1.6 \pm 0.2	6.9
	6	3.6 \pm 1.0	15.3
	12	5.6 \pm 1.0	23.6
	24	7.3 \pm 0.9	45.5
	Control (24 hrs.)	16.0 \pm 2.2	
KSCN, 10 mgm.	Control	23.7 \pm 1.6	
	1	4.4 \pm 1.3	18.5
	6	9.0 \pm 1.4	38.0
	12	12.7 \pm 1.7	53.5
	24	14.7 \pm 2.4	91.6
	Control (24 hrs.)	16.0 \pm 2.2	

TABLE 4

DRUG	DOSE IN MGM.	% OF ADMINISTERED ¹³¹ I COLLECTED BY THYROID S.E.M.		% OF CONTROL COLLECTION
Thiouracil*	None	44.0	±4.2	
	10.0	1.2	±0.1	2.7
	5.0	1.4	±0.2	3.2
	1.0	25.4	±5.1	57.7
	0.5	21.2	±3.5	48.2
	0.1	29.7	±4.9	67.5
Propyl thiouracil	None	14.0	±1.9	
	10.0	0.3	±0.05	2.3
	5.0	1.0	±0.04	7.1
	1.0	3.9	±0.08	27.9
	0.5	4.9	±1.1	35.4
	0.1	7.4	±1.3	53.1
Benzyl thiouracil	None	14.0	±1.9	
	10.0	0.4	±0.07	2.5
	5.0	0.3	±0.03	2.3
	1.0	0.4	±0.05	2.5
	0.5	1.0	±0.4	7.1
	0.1	3.1	±0.5	22.2
TC-68	None	18.0	±3.0	
	5.0	1.4	±0.4	7.5
	1.0	7.0	±1.3	39.0
	0.1	25.3	±3.1	140.0
TC-105	None	16.0	±2.7	
	10.0	4.8	±0.8	30.1
	5.0	6.9	±1.5	43.4
	1.0	13.1	±1.1	82.1
	0.5	12.2	±1.7	76.2
	0.1	8.2	±1.1	51.4
KSCN	None	13.7	±1.9	
	25.0	0.1	±0.01	0.5
	10.0	1.7	±1.0	12.2
	5.0	1.8	±0.1	12.8
	1.0	6.0	±0.9	43.1
	0.1	13.2	±1.2	96.2
Aminothiazole	None	16.0	±1.5	
	10.0	0.4	±0.03	2.3
	5.0	2.6	±0.6	16.4
	1.0	11.3	±1.11	70.8
	0.5	15.4	±1.8	96.8
	0.1	18.0	±2.1	113.0

* From Larsen, Keating, Peacock and Rawson.

TC-68 and methylthiouracil, escape was clearly evident by 8 hours. In cockerels there was definite evidence of escape from the block produced by minimal effective doses six hours after administering the test drugs. With the exception of aminothiazole the escape was almost complete by 24 hours.

In comparing the goitrogenic effects of these drugs as observed by Astwood and associates (5) with the results herewith reported it becomes apparent that the goitrogenic action of these agents is not entirely dependent upon the inhibition to

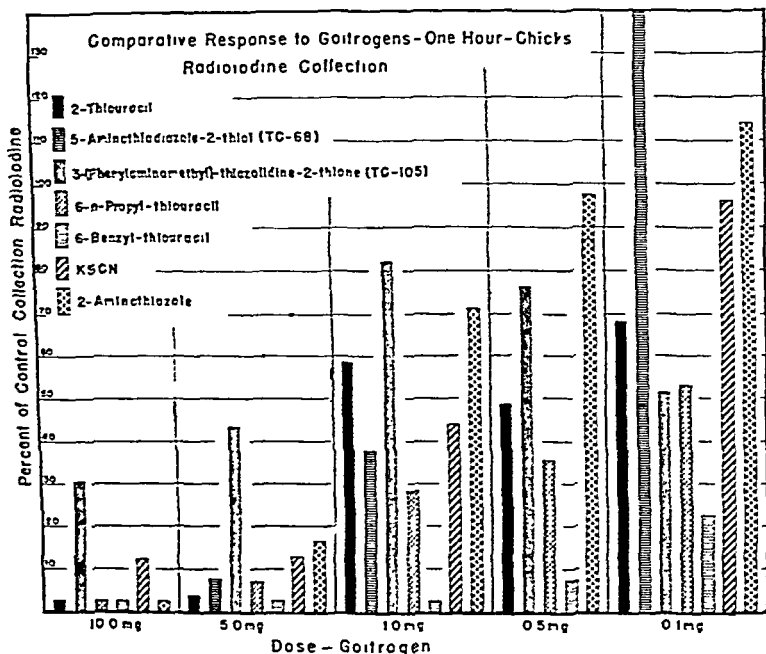


FIG. 2 SHOWING COLLECTION OF RADIOACTIVE IODINE BY THYROIDS OF CHICKS INJECTED WITH VARYING DOSES OF CERTAIN GOITROGENIC COMPOUNDS ONE HOUR EARLIER

iodine collection. Indeed in the light of the observations reported by Albert, Rawson, Merrill, Lennon and Riddell (7), who found that most of these goitrogenic agents when incubated with thyrotropic hormone, act to increase the thyroid stimulating effect, these studies suggest that the goitrogenic action of these agents may in part be due to some other mechanism such as an augmenting effect on the animals' own thyroid stimulating hormone.

It is suggested therefore that the methods herewith described would provide a more accurate method of studying the antithyroid activity of various goitrogenic drugs. In view of the discrepancies obtained in the two species used in this

thyroid effect which is about 10 times greater than that of thiouracil and methylthiouracil. These figures are consistent with the comparative goitrogenic effects of these drugs observed in chronic feeding studies. However, TC-68 and TC-105, two agents which in chronic feeding experiments have been observed to be slightly more goitrogenic than thiouracil are only about one-tenth as active in inhibiting the collection of radioiodine.

In the chick all of these agents were observed to produce a block to the collection of iodine. However, in all instances the dose of drug required to inhibit the

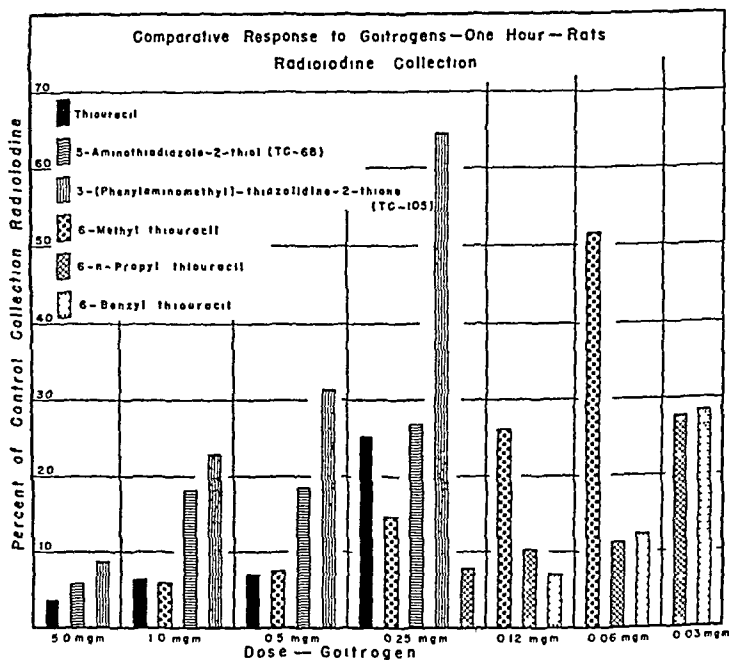


FIG. 1. SHOWING COLLECTION OF RADIOACTIVE IODINE BY THYROIDS OF RATS INJECTED WITH VARYING DOSES OF CERTAIN GOITROGENIC COMPOUNDS ONE HOUR EARLIER

thyroid's capacity to collect and concentrate iodine in this species was observed to be considerably greater than that in the rat.

In the chick, propylthiouracil was no more active than thiouracil whereas benzylthiouracil had 10 times greater activity than either thiouracil or propylthiouracil. TC-68 had the same degree of activity as thiouracil whereas TC-105, potassium thiocyanate and aminothiazole were about one-half as active.

The duration of effective block produced by the minimal effective doses of these agents in the rat was in the neighborhood of 8 hours, though in the cases of

In the cockerel, the minimal effective inhibitory doses of all compounds were greater than in the rat. Benzylthiouracil was observed to be approximately ten times more effective than thiouracil, propylthiouracil and TC-68. TC-105 aminothiazole and potassium thiocyanate had about one-half the activity of thiouracil. The duration of action of minimal effective doses was six hours. With the exception of aminothiazole, in all instances escape from the block to iodine collection was complete 24 hours after injection of the drug.

Certain fundamental differences between the iodine-blocking effect and the goitrogenic action of these agents are discussed.

REFERENCES

1. RAWSON, R. W., TANNHEIMER, J. F., AND PEACOCK, W.: *Endocrinol.*, **34**: 245, 1944.
2. FRANKLIN, A. L., LERNER, S. R., AND CHAIKOFF, I. L.: *Endocrinol.*, **34**: 265, 1944.
3. LARSON, R. A., KEATING, F. R., JR., PEACOCK, W., AND RAWSON, R. W.: *Endocrinol.*, **36**: 149, 1945.
4. LARSON, R. A., KEATING, F. R., JR., PEACOCK, W., AND RAWSON, R. W.: *Endocrinol.*, **36**: 160, 1945.
5. ASTWOOD, E. B., BISSELL, A., AND HUGHES, A. M.: *Endocrinol.*, **37**: 456, 1945.
6. VANDERLAAN, J. E., AND VANDERLAAN, W. P.: *Endocrinol.*, **40**: 403, 1947.
7. ALBERT, A., RAWSON, R. W., MERRILL, PRISCILLA, LENNON, BEATRICE, AND RIDDELL, CHARLOTTE: *Endocrinol.*, **40**: 299, 1947; **40**: 303, 1947; **40**: 361, 1947.
8. STANLEY, M. M., AND ASTWOOD, E. B.: *Endocrinol.*, **41**: 66, 1947.

study, it seems quite likely that any agents to be used therapeutically in controlling thyroid function should be assayed in a similar manner in humans. This method has been used by Stanley and Astwood (8).

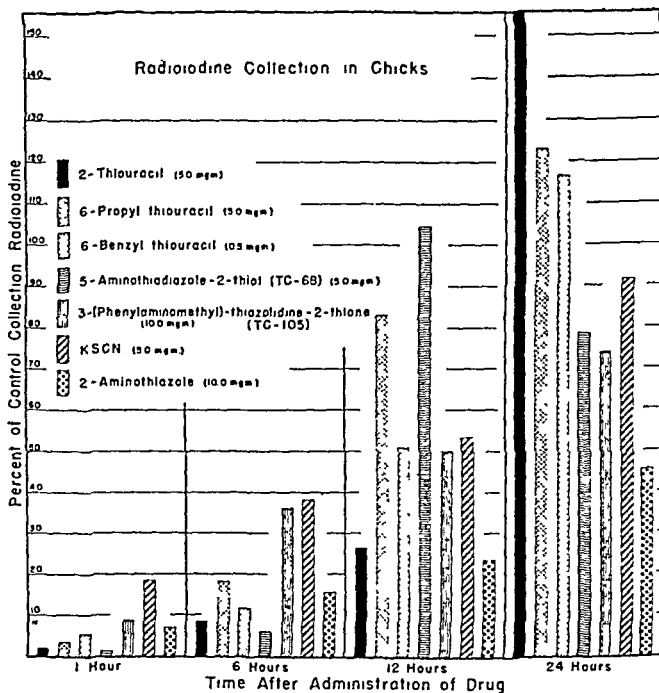


FIG. 3 SHOWING COLLECTION OF RADIOACTIVE IODINE BY CHICK THYROIDS AT VARIOUS INTERVALS AFTER ADMINISTRATION OF THE MINIMAL DOSE OF GOITROGEN CAUSING MAXIMAL BLOCK TO RADIOIODINE COLLECTION AT ONE HOUR

SUMMARY

The antithyroid action of several goitrogenic compounds was studied quantitatively by comparing the effect and duration of effect of varying doses of these agents, when injected subcutaneously, on the collection of radioiodine by the thyroids of rats and chicks.

The drugs studied included thiouracil, propylthiouracil, benzylthiouracil, methylthiouracil, 5-aminothiadiazole-2-thiol (TC-68), 3-(phenylaminomethyl)-thiazolidine-2-thione (TC-105), 2-aminothiazole and potassium thiocyanate.

In the rat, it was observed that propyl and benzylthiouracil were ten times as effective as thiouracil and methylthiouracil in inhibiting the thyroid's natural capacity to collect iodine. The duration of inhibitory action of minimal effective doses of these drugs was not materially different. In all cases at least partial escape from the drug effect occurred eight hours after administration.

Reid Hunt

1870-1948

Reid Hunt, Emeritus Professor of Pharmacology at the Harvard Medical School died on March 7, 1948 after a long illness. A charter member of the American Society for Pharmacology and Experimental Therapeutics, he served as its first secretary and third president and continued his active interest throughout his life by contributing his wise counsel to the affairs of the Society.

Doctor Hunt was born in Martinsville, Ohio April 20, 1870 and received his early education in the schools and colleges of his native state and his baccalaureate from Johns Hopkins in 1891. It was during his first stay at the Hopkins that he came under the influence of Newell Martin and received the coveted position as his student assistant. This influence was to be enduring and throughout Hunt's life his interest in the circulation never flagged. He went from Hopkins to Bonn to work with Binz for a year before returning to work for his Doctorate in Physiology under Howell; this degree he received from Hopkins in 1896 simultaneously with the M.D. from the University of Maryland. In the summer of 1896 he went to Chicago for chemical study with Jacques Loeb and Stieglitz. While there he isolated a veratrin-like alkaloid from poisonous plants that were killing cattle on the Western ranges. In the autumn of this year he became Tutor in Physiology at Columbia University. In the next two years he completed his study of the cardiac nerves which has remained as a classic in Physiology.

On completion of the two years at Columbia he turned definitely to pharmacology as a career by accepting a position with Abel at Hopkins. Then in 1902 he went to Ehrlich's laboratory in Frankfurt where he worked until 1904. This time with Ehrlich was very happy and fruitful. He imbibed Ehrlich's enthusiasm for chemotherapy and began the work on cyanides that was to lead to the discovery of the Acetonitrile Test for thyroid activity, later generally known as the Reid Hunt Reaction. His work on the activity of thyroid preparations was continued after his return from Frankfurt in 1904 to assume the position of Chief of the Pharmacological Division of the Hygienic Laboratory. Two very important findings grew out of this work: the first demonstration of the presence of the thyroid hormone in the blood and the proof that the activity of thyroid preparations is proportional to the iodine content thereof. His assumption of the post of Chief Pharmacologist at this time was very important for American medicine. The enforcement of the Pure Food and Drug Laws rested largely on the work performed in his laboratory where the case against patent medicines was developed from the scientific point of view. During this period he made his brilliant descriptions of the pharmacology and toxicity of methyl and ethyl alcohols. These studies became of great importance during the prohibition period.

During his tenure at the Hygienic Laboratory he began his researches on the



Reid Hunt

EFFECTS OF AMPHETAMINE AND COCAINE ON NEUROMUSCULAR FUNCTION¹

J. V. LUCO, C. EYZAGUIRRE AND F. PEREZ

*Department of Pharmacology and Biochemistry, Catholic University of Chile,
Santiago, Chile*

Received for publication October 22, 1947

Various pharmacological actions of amphetamine (benzedrine or phenyl-1-propylamine) on the central nervous system (1), on smooth muscle (2), and on cardiac and neuromuscular excitability (3) suggested to us that this drug might have pharmacological effects on neuromuscular function. A chemical and pharmacological similarity between amphetamine and epinephrine suggested that amphetamine might also be a de-fatiguing drug.

The custom of some South American Indians of chewing coca leaves (*Erythroxylum coca*) to diminish fatigue is well known. However, the mechanism of that effect is not well understood.

Most of the literature deals with the depressive effect of cocaine on the neuromuscular mechanism: MacGregor (4), Jaco and Wood (5) and Bülbring and Burn (6) reported depression from cocaine, although the latter also observed an increase in muscle response.

The reason for combining in one article the results of the study of these two drugs was the similarity noted in their effects.

METHOD. Cats were anesthetized with nembutal-urethane (nembutal 0.3 grams in 1 cc. of a 25% urethane solution; 1 cc. per kgm. injected intraperitoneally). The muscles studied were the auriculars, the rectus superior of the eye, the gastrocnemius-plantaris-soleus, the anterior tibials and quadriceps. Muscles were stimulated to a maximum by short pulses of 0.5 milliseconds; frequency was controlled by electronic valves. For indirect stimulation electrodes were shielded silver wires; for direct stimulation steel needles were inserted in the muscles and tendons. (Muscles were denervated by aseptic section 4 to 10 days prior to the experiment.) Direct stimulation was also performed during curarization with Intocostin or beta erythroidine. The leg muscles were fixed by drills in the tibia or femur. In studying the facial muscle, the head was held by a Czermak clamp.

In some experiments the denervated muscles were stimulated by an injection of acetylcholine into the abdominal aorta or into the carotid artery. Doses employed varied from 4 to 20 micrograms in a volume not exceeding 0.20 cc.

Muscle contractions were recorded on a kymograph. To determine nerve action potentials, the sciatic nerve was isolated near the pelvis. The peroneal nerve was cut near the fibula and the central end was dissected free for about 3 cm. Stimulations were done at an average frequency of 30 per second, with one pair of shielded silver-wire electrodes being placed at the pelvic end of the peroneal nerve segment. In order to confine the stimulus the popliteal and nerves to the hamstring were cut. Recording electrodes of the Sherring-

¹ Aided by a grant for research from the Ella Sachs Plotz Foundation, Boston, Mass., and from the Gildemeister Foundation, Santiago, Chile.

Grateful acknowledgment is made to Squibb & Sons, New York, for the Intocostin and to Hoffmann-La Roche, Inc., New Jersey for the Acetylcholine.

choline compounds, the idea for which may have been germinating since his days with Abel when he noted the depressor effects of choline in the residues from adrenal extracts. His demonstration of the amazing activity of acetylcholine led to the prompt identification of Loewi's "Vagusstoff" as none other than this compound and so to our knowledge of the humoral transmission of parasympathetic impulses.

In 1913 Doctor Hunt was called to the Professorship of Pharmacology at Harvard Medical School and continued in this post until his retirement as Emeritus Professor in 1935. Here he completed his work on the cholines and studied the quarternary ammonium compounds from the same point of view, that of the relation between chemical constitution and physiological action. In addition he made notable contributions to the development of pure arsphenamine.

During this period he was very active in his campaign for a better and more intelligent use of drugs in the clinic. A member of the Council on Pharmacy and Chemistry of the American Medical Association since its organization he became Chairman in 1925 and held this post until his resignation in 1935. The medical profession owes him an immense debt for his sound work on this body which under his guidance became the potent force for the ethical exploitation of new drugs. At the same time improvements in the United States Pharmacopoeia developed under his presidency of the Pharmacopoeial Convention from 1920 to 1930. During this period he was also a member of the Drug Standardization Committee of the League of Nations.

Doctor Hunt was honored by membership in many scientific societies both here and abroad including the National Academy of Sciences, the American Academy of Arts and Sciences, the Association of American Physicians, the American Chemical Society (of which he was chairman of the Northeastern Section), the Leopold Carol Akademie, the Deutsche Pharmakologische Gesellschaft and many others. He was Consultant to the Chemical Warfare Service of the United States Army, to the United States Public Health Service and the Massachusetts Department of Public Health.

He was personally shy and modest, tolerant in his opinions but uncompromising in his scientific standards. He had no use for sham or overdramatization of scientific work. His own experiments were carefully thought out and planned in detail and were performed with superb operative technique and skill. He was at his best in discussions among small groups where his charm of person and personality was potently felt by all who had the privilege of such association. Those who knew him will cherish his memory and his life and contributions will be a continuing source of inspiration to keep high the standards of American pharmacology.

G. PHILIP GRABFIELD

the dose of acetylcholine was greater, it produced depression (figs. 1 and 8). If amphetamine produced muscular shortening under sub-maximal tension, or when maximal tension was subsiding, acetylcholine produced first a contraction and then a decrease in the response from amphetamine.

The action of amphetamine during indirect stimulation was noted. Amphetamine was injected into the abdominal aorta in a dosage of 0.125 to 5 mg. The nerves of the quadriceps, anterior tibials and gastrocnemius-plantaris-soleus were stimulated to the maximum with frequencies of 0.5 to 60 per second.

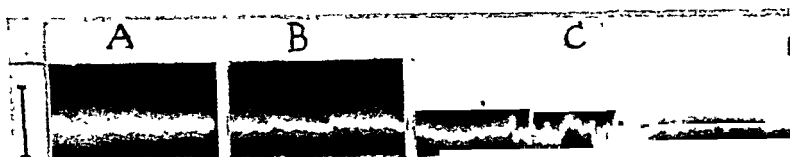


FIG. 2. Action of amphetamine on muscle electrogram of rectus superior of eye (previous acute section of 3rd pair). Time: one second. Calibration: 500 microvolts. A is normal electrogram of muscle; B is after 10 mg. of intra-arterial amphetamine; and C immediately after B and response to 40 micrograms of acetylcholine intra-arterially.

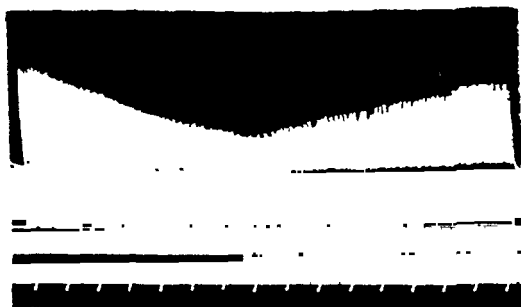


FIG. 3. Action of amphetamine on normal fatigued gastrocnemius-plantaris-soleus. Frequency of 1.5 per second. Upper signal shows indirect stimulation; middle indicates intra-arterial injection of 5 mg. of amphetamine; lower, 1 minute intervals.

Effects differed according to the frequency and degree of fatigue of the muscle. With a low frequency of about 0.5 per second, if the muscle were not fatigued by contraction (10) the drug had no effect. If the muscle were fatigued, amphetamine in doses up to 1 mg. produced stronger muscular contractions—an evident de-fatiguing effect was observed (fig. 3). When muscles were stimulated with higher frequencies, intra-arterial amphetamine always decreased muscular contractions (fig. 4).

The action of amphetamine on direct muscular stimulation was as follows: with frequencies of 0.5 to 5 per second, the drug had no effect on unfatigued muscles, but a clear de-fatiguing effect was produced on a tired muscle (fig. 5); with frequencies of more than 10 per second depression of muscular contraction

ton type were applied to the dissected peripheral end of the peroneal nerve. Blood vessels to the nerve (7) were preserved. Electric responses were recorded from a cathode-ray oscillograph.

Spikes of A fibers were studied with the aid of a five stage capacity coupled amplifier. Alpha wave decrease was used as the criterion of block. Muscle response was recorded by placing a concentric electrode (used by Adrian and Bronk (8)) in the body of the muscles.

RESULTS. A: Effect of Amphetamine. We observed the contractile response after amphetamine. When the drug was injected intra-arterially in doses larger than 0.5 mg., it produced a contractile response in striated muscles (fig. 1).

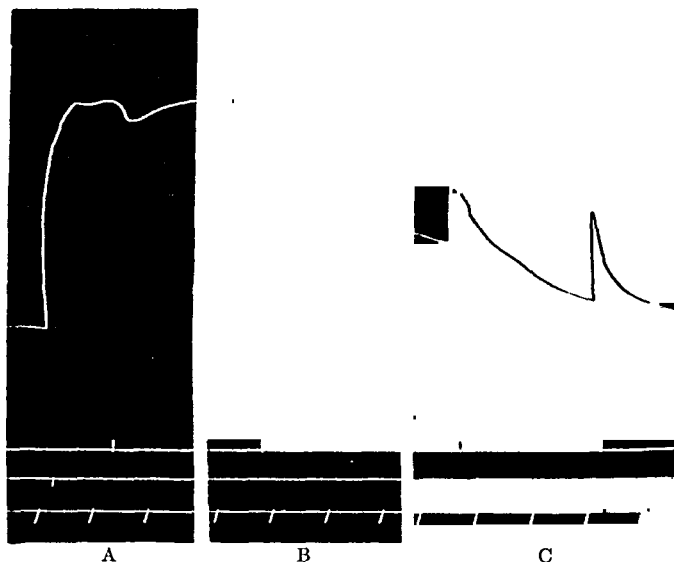


FIG. 1. Action of amphetamine on the rectus superior of the eye (previous acute section of 3rd pair) after 40, 80, 40, 40 micrograms respectively of intra-arterial acetylcholine. Upper signal denotes injection of acetylcholine; middle, the injection of 5 mg. of intra-arterial amphetamine; lower, 60 second intervals. Three minutes between A and B, 10 minutes between B and C.

This response was constant in eye muscles, more or less frequent in denervated muscles and only observed once in normal muscles. Muscle shortening was sluggish and persistent, similar to the effect obtained with veratrine (9). In one experiment contraction lasted for about 20 minutes. Amphetamine was injected while the muscle electric response was recorded and no effect was observed (fig. 2). The response to amphetamine is a contracture since there is no accompanying electric response.

When acetylcholine was injected during amphetamine contracture, two effects were observed: if the injection was done when amphetamine had produced the maximum contracture, a small dose of acetylcholine was without effect; but when

was always observed, more evident when the dose of amphetamine employed was greater. Amphetamine in small doses of 0.25 mg. depressed the normal muscle indirectly stimulated, and did not have any effect upon the denervated muscle directly stimulated, although a larger dose of more than 0.5 mg. decreased the response of the latter (fig. 6).

We also observed the action of amphetamine upon the gastrocnemius-plantaris-soleus, quadriceps and auricular muscles stimulated by acetylcholine. Four to 10 micrograms of acetylcholine were injected intra-arterially every one or two

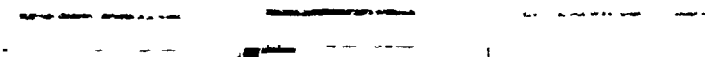


FIG. 6. Action of amphetamine on normal gastrocnemius-plantaris-soleus muscles. Frequency of 32 per second. Upper signal shows stimulation; middle intra-arterial injection respectively of 0.125 mg., 0.25 mg. and 1 mg. of amphetamine; lower signal indicates 1 minute intervals. Ten minutes between A, B, and C. Upper record shows direct stimulation of muscles (denervated 8 days prior); lower record shows indirect stimulation in other leg.

minutes; when the muscular response was constant, amphetamine was injected one minute before the acetylcholine. The results showed that amphetamine increased the response to acetylcholine (fig. 7). If amphetamine *per se* determined the response, muscular shortening induced by acetylcholine was equal or less than that existing before the injection of amphetamine (fig. 8). Depression might be due to amphetamine contracture.

Amphetamine, in small or large doses, did not modify the electrogram of motor nerves either under maximum or sub-maximum stimulation.

B: Effect of Cocaine.

Action of cocaine on the response to indirect stimulation: Cocaine was injected

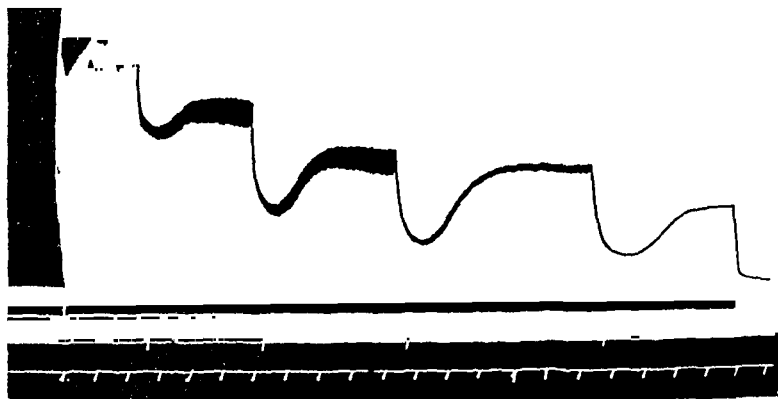


FIG. 4. Action of amphetamine on normal non-fatigued gastrocnemius-plantaris-soleus. Frequency of 10 per second. Upper signal shows indirect stimulation; middle the intra-arterial injection of 5 mg. of amphetamine; lower, 1 minute intervals.



FIG. 5. De-fatiguing action of amphetamine on arteri (curarized with intocostin). Frequency of 15 per second. stimulation (control period of indirect stimulation shown by 1.5 mg. of intra-arterial amphetamine; lower, 1 minute intervals.

was always observed, more evident when the dose of amphetamine employed was greater. Amphetamine in small doses of 0.25 mg. depressed the normal muscle indirectly stimulated, and did not have any effect upon the denervated muscle directly stimulated, although a larger dose of more than 0.5 mg. decreased the response of the latter (fig. 6).

We also observed the action of amphetamine upon the gastrocnemius-plantaris-soleus, quadriceps and auricular muscles stimulated by acetylcholine. Four to 10 micrograms of acetylcholine were injected intra-arterially every one or two

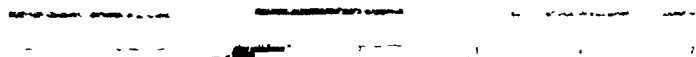


FIG. 6. Action of amphetamine on normal gastrocnemius-plantaris-soleus muscles. Frequency of 32 per second. Upper signal shows stimulation; middle intra-arterial injection respectively of 0.125 mg., 0.25 mg. and 1 mg. of amphetamine; lower signal indicates 1 minute intervals. Ten minutes between A, B, and C. Upper record shows direct stimulation of muscles (denervated 8 days prior); lower record shows indirect stimulation in other leg.

minutes; when the muscular response was constant, amphetamine was injected one minute before the acetylcholine. The results showed that amphetamine increased the response to acetylcholine (fig. 7). If amphetamine *per se* determined the response, muscular shortening induced by acetylcholine was equal or less than that existing before the injection of amphetamine (fig. 8). Depression might be due to amphetamine contracture.

Amphetamine, in small or large doses, did not modify the electrogram of motor nerves either under maximum or sub-maximum stimulation.

B: Effect of Cocaine.

Action of cocaine on the response to indirect stimulation: Cocaine was injected

intra-arterially in doses of 0.125 to 4 mg. The nerve was stimulated with frequencies varying from 0.5 to 60 per second. Cocaine with a low frequency stimulation of less than 10 per second (if contraction fatigue as defined by del

A

B

FIG. 7. Action of acetylcholine on quadriceps (denervated 7 days prior) before and after amphetamine. Upper signal shows intra-arterial injection of 0.5 mg. of amphetamine; middle shows intra-arterial injection of 10 micrograms of acetylcholine every 2 minutes; lower, 1 minute intervals. Four minutes between A and B.

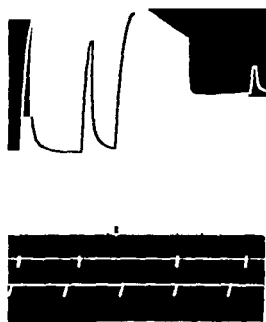


FIG. 8. Action of acetylcholine on auricular muscles (denervated 6 days prior). Upper signal indicates injection of 1 mg. of amphetamine in carotid artery; middle signal shows injection every 2 minutes of 4 micrograms of acetylcholine; lower indicates 1 minute intervals. Note amphetaminic contracture and depression produced by acetylcholine (injected during contracture).

Pozo (10) were not present) depressed the response, as was reported by MacGregor and by Jaco and Wood. Smaller doses of cocaine produced depression with higher frequency of stimulation.

The de-fatiguing action of cocaine described by Bülbring and Burn (6) was

also confirmed. Short muscles injected with cocaine and under stimulation at a frequency between 20 and 60 per second, for a period of 5 seconds of rest, showed three types of response. With low doses of 0.125 mg. of cocaine either no effect was observed, or a strengthening of muscular contractions appeared after a short period of depression of variable intensity (fig. 9). The increased response was most apparent during the first stimuli. With doses up to 0.5 mg. depression was also observed. Stronger muscular contractions appeared at the beginning of tetanus, although at the end a depression was observed (fig. 10). With cocaine doses higher than 1 mg., a depression resulted.

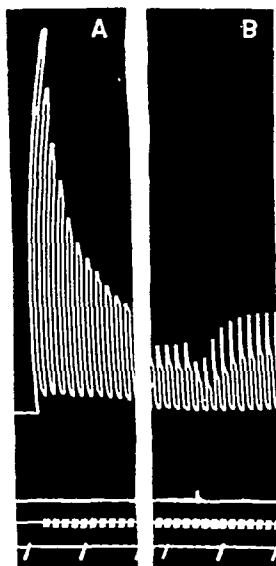


FIG. 9 Indirect interrupted stimulation of auricular muscles at a frequency of 22 per second. Upper signal denotes injection of 0.125 mg. of cocaine in carotid artery; middle indicates 5 seconds of stimulus, 5 seconds of rest; lower, 1 minute intervals. Four minutes between A and B.

Action of cocaine on the response to direct stimulation: When unfatigued gastrocnemius-plantaris-soleus muscles were stimulated with single shocks, cocaine injected into the abdominal aorta in doses of 0.25 mg. to 1 mg. did not modify the response. When the doses were increased to 5 mg. the drug produced some depression. When fatigue appeared and cocaine was injected in 5 mg. doses, some increased contraction was noted (fig. 11). At frequencies of 10 to 60 per second, the same dose decreased muscle response, and to a greater extent when the dose was greater. If a normal muscle, previously curarized with beta-erythroidine, was directly stimulated, cocaine injections brought about the same effects as on denervated muscles (fig. 12).

Action of cocaine on muscles stimulated with acetylcholine: The gastrocnemius-plantaris-soleus, quadriceps and auricular muscles were used, having been de-

nervated 4 to 10 days before the experiment. Acetylcholine was injected intra-arterially every two minutes in doses of 4 to 10 micrograms. When the muscular responses were constant, cocaine was injected one minute before the acetylcholine. Results showed that cocaine increased or decreased muscle response to

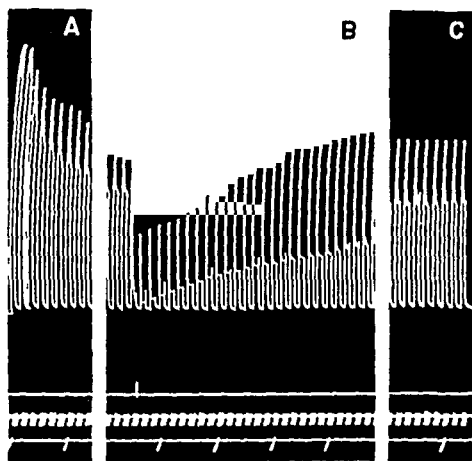


FIG. 10. Indirect stimulation of auricular muscles at a frequency of 20 per second. Upper signal denotes injection of 0.50 mg. of cocaine in carotid artery; middle indicates 5 seconds of stimulus, 5 seconds of rest; lower, 1 minute intervals. Fifty seconds between A and B, 6 minutes between B and C.

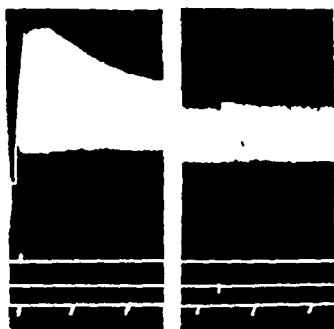


FIG. 11. Direct stimulation at a frequency of 4 per second of gastrocnemius-plantaris-soleus, denervated 7 days prior. Upper signal indicates first stimulus; middle shows injection of 5 mg. of cocaine into aorta; lower, 10 seconds intervals. Three minutes between A and B.

acetylcholine, the difference depending on the amount of cocaine employed: small doses up to 0.5 mg. increased the response; greater doses of 2 mg. or more decreased the response (fig. 13).

Action of cocaine on nerve action potentials: We also tried to observe the classic nerve blockage by cocaine. With stimulation at a frequency of 30 per second

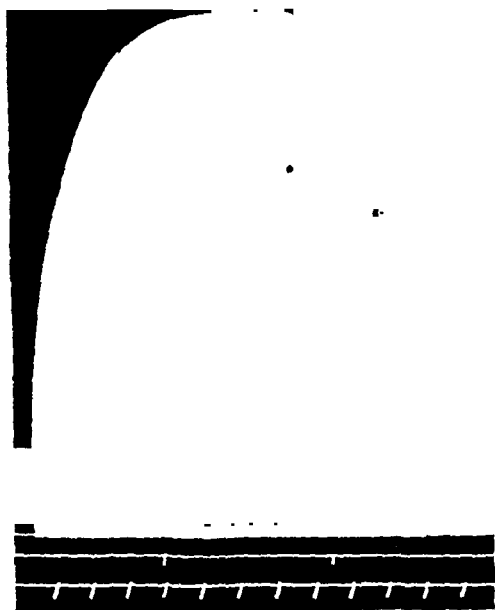


FIG 12 Direct stimulation at a frequency of 20 per second of gastrocnemius-plantaris soleus (curarized with beta-erythroidine) Upper signal shows period of stimulus; middle, the injection of 0.50 mg and 1 mg of cocaine into abdominal aorta; lower, 10 second intervals

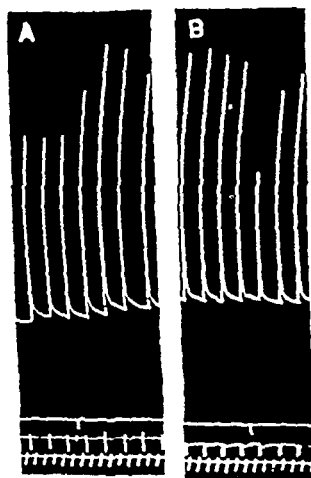


FIG 13 Effect of cocaine on muscular contractions induced by acetylcholine (quadriceps previously denervated) Upper signal denotes injections (45 minutes apart) of 0.5 mg and 2 mg of cocaine in abdominal aorta, middle shows 10 micrograms of acetylcholine injected in abdominal aorta every 2 minutes, lower, 1 minute intervals

during 5 to 10 seconds, with 5 to 10 seconds of rest, depression of the spike was observed when doses were higher than 1 mg. After cocaine, the height of the spike during the first stimuli was normal but soon decreased (11).

Action of cocaine on muscle action potentials: Low frequencies of 1 to 5 per second were used. The cocaine doses were between 1 mg. and 5 mg. With small doses of 1 mg. there was some depressive effect. This effect was greater when the dose was larger. A decrease in muscle tension paralleled the action potentials.

DISCUSSION. The effect of amphetamine and of cocaine might be related to the axone, to synaptic transmission or to the contractile mechanism. The effect of the drugs upon the electrogram of motor nerves was studied in connection with the axone; the action of the drugs at different frequencies and muscular response to acetylcholine was studied in connection with the synaptic mechanism; and amphetamine and cocaine was injected during direct stimulation of denervated muscles to enable us to study the muscular mechanism.

Amphetamine had two effects on the neuromuscular system: it both decreased and increased function, phenomena fundamentally determined by physiological conditions during administration of the drug. These effects had no relation to axone conductivity.

The lack of increase of normal activity of the unfatigued muscle under low frequency stimulation demonstrated that amphetamine did not affect the normal mechanism, but restored to normal activity the fatigued mechanism.

During contraction fatigue amphetamine had a de-fatiguing effect.

Depression seemed to be produced by the administration of amphetamine during intense activity of the contractile mechanism. If high muscle tension developed (with direct or indirect stimulation, see fig. 6), the drug produced depression; if tension was low, no effect was observed unless contraction fatigue was present.

Response to amphetamine *per se* was more frequently observed in denervated muscles than in normal ones, possibly an example of Cannon's law of denervation (12). On the other hand, eye muscles, which are most sensitive to acetylcholine, were also most sensitive to amphetamine.

The tachyphylactic effect of amphetamine on blood pressure is well known (2); however, tachyphylaxis was not present in the muscle when the contractile response or depression during muscle activation was tested. It appears that tachyphylaxis does not depend alone on the quality of the drug, but also on the effector.

Similarly to amphetamine cocaine had two effects on the neuromuscular system: it both decreased and increased function. The increase in function was observed in three groups of experiments: when fatigue was produced by slow direct stimulation; when fatigue was produced by slow indirect stimulation; and when fatigue was brought about by short tetanus interrupted with rest periods. Under these three conditions fatigue was probably related to contraction fatigue. Under the latter condition some transmission fatigue might have been present,

but contraction fatigue prevailed when a slow frequency of 20 per second was used.

If, as is probable, contraction fatigue was due to exhaustion of energy, the action of cocaine would be related to a restoration of energy. This effect is quite analogous to the defatiguing action of amphetamine.

The depressive effect of cocaine appeared under direct or indirect stimulation; intensity depended on the dose and on the frequency of stimulation. With a high frequency of 30 to 60 per second, a small dose of cocaine produced intense depression; with a single shock only large doses decreased response.

As was observed with amphetamine, a high tension seemed an important preliminary to the appearance of cocaine depression. Depression also might be related to the effect of the drug on nerve conduction, but doses necessary to depress nerve action potentials, under the experimental conditions, were higher than the doses necessary to depress muscle response.

Effects observed in muscles indirectly stimulated with frequencies of 20 to 30 per second during an interval of 5 to 10 seconds, with equal rest periods, could be similarly explained.

As a control measure, blood pressure was registered in some experiments. Blood pressure modifications were not related to the muscular effects of the two drugs.

The increase in muscle response induced by cocaine did not differ greatly in cats with adrenal glands removed.

SUMMARY

Cats anesthetized with nembutal were used in studying the effect of amphetamine and of cocaine on action potentials of A fibers, on neuromuscular transmission and on muscular contractions. No important effect of amphetamine was observed upon the alpha waves of the A fibers.

Amphetamine has a de-fatiguing effect during low frequency stimulation. During high frequency direct or indirect stimulation (*i.e.*, during intense activity of the contractile mechanism), the drug acted as a depressant.

The response of denervated muscles to acetylcholine was increased by amphetamine. The drug produced a contractile response in both normal and denervated skeletal muscles. Since this response was not accompanied by action potentials, it was a contracture.

Although amphetamine had a tachyphylactic effect on blood pressure, apparently it had none on muscular response.

During indirect muscular stimulation, cocaine acted to increase or decrease response. The same effect was produced when a denervated or fully curarized muscle was directly stimulated. The more tension developed by the muscle, the easier it was to demonstrate depression. Increased response seemed to be related to the development of contraction fatigue. Cocaine in small doses increased the response of denervated muscles stimulated by acetylcholine. With large doses a depression resulted.

REFERENCES

1. SCHULTE, J. W., REIF, E. C., BACHER, J. A., LAWRENCE, W. S., AND TANTER, M. L.: THIS JOURNAL, 71: 62, 1941.
2. DETRICK, L. E., MILLIKAN, R., MODERN, F. S., AND THIENES, C. H.: THIS JOURNAL, 60: 56, 1937.
3. GÜNTHER, B.: THIS JOURNAL, 76: 369, 1942.
4. MACGREGOR, D. F.: THIS JOURNAL, 66: 350, 1939.
5. JACO, N. T. AND WOOD, D. R.: THIS JOURNAL, 82: 63, 1944.
6. BÜLBRING, E. AND BURN, J. H.: THIS JOURNAL, 68: 150, 1940.
7. ACHESON, G. H. AND ROSENBLUETH, A.: Am. J. Physiol., 133: 736, 1941.
8. ADRIAN, E. D. AND BRONK, D. W.: J. Physiol., 67: 119, 1929.
9. BACQ, Z. AND GOFFART, M.: Arch. int. Physiol., 49: 189, 1939.
10. POZO, E. C. DEL.: Am. J. Physiol., 135: 763, 1942.
11. LORENTE DE NÓ, R.: J. Neurophysiol., 2: 402, 1939.
12. CANNON, W. B.: Am. J. M. Sc., 198: 737, 1939.

THE EFFECT OF QUANTITATIVE AND QUALITATIVE PROTEIN DEFICIENCY ON TOLERANCE TO EMETINE

K. GUGGENHEIM AND EDITH BUECHLER

Department of Hygiene and Bacteriology, The Hebrew University, Jerusalem

Received for publication February 26, 1948

The influence of the nutritional status on drug tolerance seems to be established for many drugs. Particularly the protein content of the diet appears to be of primary importance in determining the susceptibility to poisoning with various drugs. Goldschmidt, Vars and Ravdin (1) found with rats and Miller and Whipple (2) with dogs, that dietary protein diminishes the extent of the liver injury produced by chloroform. High protein diets were found to decrease the toxicity of arsphenamine (Schiffrin 3; Messinger and Hawkins 4), trinitrotoluene (Himsworth and Glynn 5), p-aminoazobenzene, dimethylaminoazobenzene (Smith, Lillie and Stohlmann 6) and selenium (Smith and Stohlmann 7; Anderson, Poley and Moxon 8). Low protein diets were found to increase the susceptibility of rats to thiourea (Dicke and Richter 9), alphanaphthylurea (Byerrum and Du Bois 10) and benzene poisoning (Li and Freeman 11; Li, Freeman and Gunn 12). The carcinogenicity of butter yellow also apparently depends upon the protein content of the diet (Kensler, Sugiura, Young, Halter and Rhoads 13).

The high toxicity of emetine severely hampers its general and intensive use in spite of its great value in the treatment of amoebic infections. Hence knowledge of the relation of diet to emetine tolerance may be of practical importance in the treatment of the disease. Brueckmann and Wertheimer (14) showed that protein deficiency lowers the resistance of rats to the drug. The present paper reports experiments with rats designed to determine: (1) the effect of various levels of dietary protein on emetine tolerance; (2) the rôle played by various animal and vegetable proteins in protecting the organism against emetine poisoning; (3) the effect of a high protein diet on the susceptibility of protein depleted rats to emetine poisoning.

METHODS. All the rats used were males; after weaning they were put on the experimental diets listed below in order to test the effect of various levels of dietary protein on emetine tolerance (grams per 100 grams ration):

	DIET C ₁	DIET C ₂	DIET C ₁₁
Casein	3	9	18
Rice starch . .	88	82	73
Olive oil	5	5	5
Salt mixture	4	4	4

These diets were supplemented with 0.1 mgm. thiamin, 0.2 mgm. riboflavin, 0.1 mgm. pyridoxin, 16 mgm. calcium pantothenate and 100 mgm. choline chloride per 100 grams ration. Each rat received, twice weekly, 100 I.U. vitamin A and 4 I.U. vitamin D.

In the experiments with qualitative protein deficiency the following protein sources were used: egg powder, dried meat, casein, processed soya bean flour, maize flour and peanut meal. Egg powder, dried meat, soya bean flour and peanut meal were fat extracted. The diets were prepared in the following manner: the various protein sources were incorporated in a protein-free basal diet composed of 91 per cent of rice starch, 5 per cent olive oil, 4 per cent salts, and the above mentioned vitamins, by replacing an appropriate amount of starch, so as to make the protein level of each diet 9 grams per 100 grams ration. The high-protein diet, containing 36 per cent casein, was prepared in a similar manner. All diets were isocaloric.

After the rats were kept for 4 weeks on our experimental diets, treatment with emetine was started. In order to compare the tolerance test—so far as possible—with the course of treatment in human amoebiasis, we chose repeated administration of the drug. Each rat received daily 0.3 mgm. emetine hydrochloride per 100 grams body weight subcutaneously. During the period of treatment the rats were still maintained on their previous diets, unless stated otherwise.

Tolerance was determined by noting the survival time, i.e. the time elapsed from the beginning of treatment to the death of the animals from emetine poisoning.

RESULTS. In our first series of experiments the effect of various levels of dietary protein on emetine tolerance was studied. Treatment with emetine

TABLE 1

Tolerance to emetine of rats maintained on different levels of dietary protein

LEVEL OF DIETARY PROTEIN (CASEIN)	NO. OF RATS	GAIN IN WEIGHT GRAMS IN 4 WEEKS	SURVIVAL TIME IN DAYS. AVER- AGE AND STANDARD DEVIATION
<i>per cent</i>			
3	38	-3	11.4 \pm 3.4
9	30	+30	14.3 \pm 3.5
18	70	+51	17.6 \pm 3.3

was started after the rats were maintained for 4 weeks on diets C₃, C₉, and C₁₈ respectively. Table 1 shows the results obtained.

As can be seen from table 1, low protein diets decrease the resistance to emetine. The effect of the different protein levels of the diets correspond, more or less, to their growth promoting effect. Statistical treatment of the survival times showed, that the three diets employed differed one from another to a highly significant degree.

In our second series of experiments the effects of various food proteins (egg, meat, casein, soya, maize, peanut—fed at 9 per cent level) on emetine tolerance were compared. All rats were paired fed in groups of 5. The results are shown in table 2.

It follows from table 2, that protein quality as well as quantity determines tolerance to emetine. Among the food proteins investigated the proteins of egg and meat rank first. Both are similar in this respect in spite of their different growth promoting qualities. Casein and soy bean proteins rank next. It is interesting to note, that soy bean protein effects the same degree of emetine tolerance as casein despite its decidedly inferior effect on growth. Maize and peanut proteins are the least effective against emetine poisoning as well as in

their growth promoting efficiency. Statistical treatment of the differences in survival time revealed the following facts: egg or meat vs. casein or soya: difference significant (probability of the occurrence of the observed mean difference in a random sample 1:50). Egg or meat vs. maize or peanut: difference highly significant (probability 1:100 or less). Casein or soya vs. maize or peanut: difference highly significant (probability 1:100 or less).

In the last series of experiments we studied the protective value of normal and high protein diets, given during the period of treatment to rats previously maintained on a low protein diet (C_3). The results are represented in table 3.

TABLE 2

Tolerance to emetine of rats maintained in different food proteins

FOOD PROTEIN	NO OF RATS	GAIN IN WEIGHT GRAMS IN 4 WEEKS	PROTEIN EFFICI- ENCY GAIN IN WEIGHT PER GRAM PROTEIN EATEN	SURVIVAL TIME IN DAYS AVER- AGE AND STANDARD DEVIATION
Egg	30	34	2.34	15.1 \pm 2.5
Meat	30	28	1.93	15.2 \pm 2.8
Casein	30	27	1.86	13.7 \pm 2.2
Soya	30	19	1.31	13.7 \pm 2.5
Maize	30	4	0.28	11.0 \pm 3.9
Peanut	30	2	0.14	11.6 \pm 3.5

TABLE 3

The protective value of normal and high protein diets against emetine poisoning of rats kept for 4 weeks on a low protein diet (8% casein)

LEVEL OF DIETARY PROTEIN (CASEIN) DURING PERIOD OF TREATMENT	NO OF RATS	SURVIVAL TIME IN DAYS. AVERAGE AND STANDARD DEVIATION
<i>per cent</i>		
3	38	11.4 \pm 3.4
18	34	17.9 \pm 3.9
36	33	18.6 \pm 2.4

It can be seen from table 3 that normal or high protein diets, fed during the period of treatment, cancel the tolerance depressing effect of the low protein diet given before the treatment. No significant difference was obtained from the use of the two protein levels (18 and 36 per cent). The increase of the protein content of the diet from 18 per cent to 36 per cent did not significantly increase the resistance of the animals.

DISCUSSION. Our experiments show, that the nutritional status of rats with respect to protein determines to a large degree their tolerance to emetine poisoning. Diets containing low protein levels or nutritionally inferior proteins decrease considerably the resistance to the drug. An 18 per cent casein diet given during the period of treatment cancels the resistance decreasing effect of a low protein diet given before the treatment. It is interesting to note that a diet

containing more than 18 per cent protein, fed during the period of treatment, does not increase the resistance to emetine poisoning above that effected by the 18 per cent protein diet.

SUMMARY

(1) Rats maintained on quantitatively and qualitatively different protein diets were treated with daily subcutaneous injections of emetine.

(2) Low protein diets decrease the tolerance to emetine.

(3) Diets containing nutritionally inferior proteins, fed at 9 per cent level, also decrease the resistance to emetine. The protection against emetine poisoning afforded by the proteins investigated was found to decrease in the following order: egg, meat, casein, soya bean, peanut, maize.

(4) A diet containing 18 per cent casein, fed during the period of treatment, cancels the tolerance depressing effect of a low protein diet, given before the treatment.

(5) A diet containing 36 per cent casein, fed during the period of treatment, did not endow the animals with additional resistance to emetine poisoning above that of the 18 per cent casein diet.

REFERENCES

1. GOLDSCHMIDT, S., VARS, H M AND RAVDIN, I S *J Clin. Inves*, **18**: 277, 1939.
2. MILLER, L L AND WHIPPLE, G H *Am J Med Sci*, **199**: 204, 1940
3. SCHIFRIN, A *Arch Path Anat*, **287**: 175, 1932
4. MESSINGER, W J AND HAWKINS, W. B *Am J Med Sci*, **199**: 216, 1940
5. HIMSWORTH, H P AND GLINN, L E *Clin Sci*, **4**: 421, 1942
6. SMITH, M I, LILLIE, R D AND STOHLNANN, E F *Pub Health Rep*, **58**: 304, 1943
7. SMITH, M I. AND STOHLNANN, E F *THIS JOURNAL*, **70**: 270, 1940
8. ANDERSON, H D, POLEY, E AND MOXON, A L *Poultry Sci*, **20**: 454, 1941
9. DICKE, S H AND RICHTER, C P *THIS JOURNAL*, **83**: 195, 1945
10. BYERRUM, R U AND DU BOIS, K P *THIS JOURNAL*, **90**: 321, 1947
11. LI, T W AND FREEMAN, V H *Am J Physiol*, **145**: 158, 1945
12. LI, T W, FREEMAN, V. H AND GUNN, I D *Am J Physiol*, **145**: 166, 1945
13. KENSLE, C J, SUGIURA, K, YOUNG, N F, HALTER, C R AND RHOADS, C. P *Science*, **93**: 308, 1941
14. BRUECKMANN, G AND WERTHEIMFR, E *Acta med orient*, **4**: 291, 1945

TISSUE DISTRIBUTION OF A TOXICANT FOLLOWING ORAL INGESTION OF THE GAMMA ISOMER OF BENZENE HEXACHLORIDE BY RATS¹

EDWIN P. LAUG²

*Division of Pharmacology, Food and Drug Administration, Federal Security Agency,
Washington 25, D. C.*

Received for publication March 6, 1948

Chemical methods for the isolation of the gamma isomer of benzene hexachloride in tissues and excreta of animals have not yet reached the stage where it is possible to determine micro quantities. Furthermore, the existing chemical methods do not differentiate between mixtures of the isomers. Considerable success attended the development of a biological assay method for DDT (1). Application of the same techniques to the determination of the gamma isomer of benzene hexachloride is reported herewith.

METHOD. With certain modifications to be described, the technique for determination of the gamma isomer was based on the bio-assay method for DDT (1).

Adult house flies, 24 to 36 hours old, were exposed to gamma isomer residues in wide-mouthed Erlenmeyer beaker flasks of 250 ml. capacity. The test flask had a flat base, the inside surface of which was about 40 (cm.)² in area. Upon this surface gamma isomer was deposited by evaporation of an ether solution containing either the standard or a tissue extract. In the case of the standards it was found necessary to add about 20 mgm. of corn oil to each flask to prevent loss of gamma isomer during and subsequent to the evaporation of the ether solution. Tissue extracts, with the exception of urine, contributed enough fat to make this precaution unnecessary. The test flasks with residues were weighed to the nearest 0.01 gram, then 100 flies were transferred to each, capped with perforated paper closures and again weighed. The flies remained in the flasks overnight for about 20 hours. At the end of this time the mortality ratio was determined. All flies still living but too helpless to walk or fly were arbitrarily counted as dead. The insects were kept in copper wire mesh storage cones and fed with diluted sucrose-sweetened milk until shortly before they were transferred to the glass exposure flask. It was found desirable also to feed the flies with sugar water at about the midpoint of their exposure period. This expedient served to maintain a low control mortality, particularly at the higher summer temperatures. Control mortality greater than 5 per cent was considered vitiating to a particular assay.

With each assay four standards and a blank were run. The standard flasks were prepared to contain 0, 1.25, 1.6 and 2.0 micrograms gamma isomer. A regression line was constructed relating log dose in mgm. per kgm. flies to "probit kill" according to the method of Bliss (2). Calculation of the unknown content of gamma isomer in the tissue extracts were then made by reference to the standard regression line. From the results of 22 assays, in which standard regression lines were constructed, the average LD 50 for the house fly was 0.72 mgm./kgm. of flies with a range of 0.56 to 0.89. The average slope of the regression line was 5.65 probits per log dose with a range of 2.2 to 8.8.

¹ A portion of the funds used in this investigation was supplied by a transfer from the Office of the Surgeon General, War Department, to the Division of Pharmacology of the Food and Drug Administration.

² Read in part before the American Society for Pharmacology and Experimental Therapeutics, Atlantic City, New Jersey, March 1948.

All assays were run in duplicate and repeated if they did not check within 25%. Recovery of gamma isomer added to tissue was also affected with an accuracy of the same order of magnitude.

In order to study the distribution of toxicant in the tissues two series of adult rats, normal males and castrate females, were fed gamma isomer of benzene hexachloride in concentration of 20, 500 and 1000 p.p.m. in the diet for periods of from 7 to 114 days. The gamma isomer was incorporated in the diet in the form of an oil solution and thoroughly mixed. No toxic symptoms were noted in any of the animals but it was observed that the castrate female rats on 1000 p.p.m. gamma isomer showed a dislike for their diet and tended to reduce their food consumption.

Extracts of the tissues were made with ether and suitable aliquots evaporated in the test flask. In conformity with previous experience (1) certain toxic reactions were elicited by the insects when they were exposed to excessive amounts of tissue extractives of liver, kidney and brain from control animals. It was therefore necessary to limit the size of the aliquot, which in turn limited the amount of gamma isomer that could be determined in the tissues of experimental animals. It was shown in recovery experiments that as long as the gamma isomer was present in concentration greater than 0.2 p.p.m. in the tissue, interference from "tissue toxicity" could be avoided. More serious was the interference of fat. Excessive amounts of this substance seemed to occlude gamma isomer and render it inaccessible to the fly. Concentrations of gamma isomer lower than 10 p.p.m. in fat were considered outside the zone of reliability for bio-assay.

RESULTS. Before applying the bio-assay test to tissue it was necessary to investigate its specificity. Flies were exposed to the alpha, beta, delta, and epsilon isomers of benzene hexachloride. It was found that it required approximately 100 to 200 times as much to effect the same kill as with the gamma isomer. In addition hepta and octachloro benzenes, which may occur as contaminants in the manufacture of benzene hexachloride, were investigated and found to have a toxicity of the same order as the hexachloro isomers. The three isomeric trichloro benzenes, possible degradation products or metabolites of benzene hexachloride, were also investigated. In this case too the toxicity to flies was of a low order. Consideration was given to the possibility of conversion of some of the alpha, beta, delta and epsilon isomers *in vivo* to the gamma isomer. To check this, the tissues of rats which had been chronically ingesting these isomers were examined. No substance toxic to flies was discovered in the tissues.

The toxic response of the house fly to the pure gamma isomer was characteristic. Furthermore, when the insects were exposed to tissue extracts from animals which had ingested gamma isomer, their reaction was indistinguishable from that caused by pure isomer. Therefore, unless other metabolites of benzene hexachloride can be characterized by chemical means, and shown also to have biological activity of a high order, it may be assumed that the toxicant in the tissues of rats ingesting gamma isomer is the gamma isomer.

Table 1 shows the storage of toxicant in the fat of the first series of rats. Measurable quantities are seen to be present in all cases even after only one week of exposure to the diet containing 20 p.p.m. gamma isomer. There is greater storage in the female castrates than in the normal males on 1000 p.p.m., and this is further emphasized when it is noted that the former consumed less gamma isomer over comparable periods of time than the latter. In this series, the exposure periods ranged up to one month only, and accumulation of toxicant with time is not especially marked.

Table 2 shows the distribution of toxicant in the tissues of the second series of rats. It can be seen that the toxicant has been found distributed in all the tissues examined, but that the maximum amounts occur in kidney and abdominal fat. In the male rat, particularly at the lower diet levels, storage of toxicant is greater in the kidney than in the fat. The reverse of this however is strikingly demonstrated in the castrate female rat. It should also be emphasized that storage of toxicant in the fat of the castrate female tends to be higher than in the male, particularly at low levels of intake.

TABLE 1

Concentration of toxicant found in the perirenal fat tissue of the rat in relation to oral ingestion of gamma isomer of benzene hexachloride

RAT NO.	SEX	PERIOD OF EXPOSURE	GAMMA ISOMER INGESTED	MICROGRAMS TOXICANT PER GRAM OF FAT
Diet contained 1000 p.p.m. gamma isomer benzene hexachloride				
		days	mgm.	
3L	F*	7	162	444
2	F*	10	171	987
1L	F*	10	192	1011
5	M	10	240	310
6K	M	10	297	530
4KL	M	30	610	333
Diet contained 20 p.p.m. gamma isomer benzene hexachloride				
7K	F*	7	3.7	20
8K	F*	30	13.5	38
9	F*	30	15.1	17
10	M	30	16.2	21
11	M	30	15.6	26
12	M	30	13.7	23

L = Liver content of toxicant in micrograms per gram. No. 1 = 3.5; No. 3 = 1.8; No. 4 = 1.7.

K = Kidney content of toxicant in micrograms per gram. No. 4 = 750; No. 6 = 183; No. 7 = 9.7; No. 8 = 11.

* = Castrate female.

A careful appraisal of the distribution in the tissues indicates that accumulation of toxicant with time does not seem to occur. Thus the four animals exposed for four months to gamma isomer did not regularly contain more toxicant in their tissues than the animals which were exposed only for one month. As a matter of fact, there appears to be a tendency for the animals on longest exposure to contain less toxicant in certain tissues; this is uniformly true for the liver, and with one exception for the kidney.

Table 3 shows the excretion of toxicant in the urine and feces of rats over a single 24-hour period taken when all of the animals had been on their respective diets for one month. During the collection period the animals had access to water but were without food in order to avoid contamination of the excreta. It

TABLE 2

Distribution of toxicant in the tissue of rats which ingested gamma isomer benzene hexachloride

RAT NO.	SEX	PERIOD OF EXPOSURE	GAMMA ISOMER INGESTED	MICROGRAMS TOXICANT PER GRAM WET TISSUE							
				Fat	Kidney	Liver	Blood	Spleen	Adrenal	Muscle	Brain
Diet contained 500 p.p.m. gamma isomer benzene hexachloride											
		days	mgm.								
13	M	32	360	226	126	1.6	0	tr	0	2.0	2.6
14	M	77	764	87	119	0.87	0.95	1.7	0	2.3	4.2
15	M	113	1214	108	114	0.66	0.51	3.3	tr	1.8	1.9
18	F*	36	307	178	33	1.5	0.37	6.9	0	4.9	6.0
17	F*	77	747	244	13	0.69	1.3	0	39	5.3	7.3
16	F*	113	939	247	15	0.64	1.2	2.8	42	4.7	4.9
Diet contained 20 p.p.m. gamma isomer benzene hexachloride											
19	M	36	15.5	tr	13	1.3	0.38	4.3	0	0	0
20	M	79	30.7	9.2	34	0.36	0.20	0	0	0	0
21	M	114	44.8	0	35	0.31	0	0	0	0	0
24	F*	36	15.7	22	5.7	0.81	0.29	1.8	0	tr	tr
23	F*	79	24.9	10	0	0.54	0	0	0	0	0
22	F*	114	40.5	14	0	0.52	0	0	0	0	0

* Castrate female.

TABLE 3

The excretion of toxicant by the rat following ingestion of gamma isomer of benzene hexachloride

RAT NO.	SEX	24-HOUR PERIOD		
		Intake of gamma isomer	EXCRETION OF TOXICANT	
			Urine	Feces
Diet contained 500 p.p.m. gamma isomer benzene hexachloride				
		avg. mgm.	micrograms	micrograms
13	M	10	9.9	tr
14	M	10	97	0
15	M	10	182	tr
18	F*	8.8	tr	11
17	F*	8.8	0	0
16	F*	8.8	0	12
Diet contained 20 p.p.m. gamma isomer benzene hexachloride				
19	M	0.40	15	0
20	M	0.40	9.2	tr
21	M	0.40	tr	0
24	F*	0.37	0	tr
23	F*	0.37	0	0
22	F*	0.37	0	tr

* Castrate female.

can be seen that excretion, varying from 0 to about 4% occurs in the urine, but peculiarly enough only in that of the males. Very small quantities only are excreted in the feces.

A number of other factors were studied in an attempt to throw some light on the metabolism of the gamma isomer. The livers of all of the animals ingesting 500 p.p.m. of gamma isomer, whether for one month or four, were found to be definitely enlarged when compared with the livers of the animals ingesting 20 p.p.m. Expressed as grams liver per kgm. rat this averaged 42 for the 500 p.p.m. diets and 31 for the 20 p.p.m., approximately a 25% enlargement. When this information became available a few preliminary *in vitro* studies with minced liver in contact with gamma isomer were made. These experiments showed approximately 25% disappearance of gamma isomer when incubated at 38° for two hours. Finally, the tissues of a number of rats, which had been on a diet containing 500 p.p.m. gamma isomer for one month, and then transferred to a control diet for three weeks, were examined. No detectable amounts of toxicant could be found in fat, liver or kidney.

DISCUSSION. Assuming that the toxicant found in the tissues is the gamma isomer, it would appear that its behavior in the rat may be described by the following pattern. (a) By comparison with DDT (3) storage in the fat is lower, but storage in the kidney higher. (b) Over short periods of at least one to four months, there does not appear to be any marked accumulation of gamma isomer in the tissues with time, as has been demonstrated for DDT (4). (c) Gamma isomer disappeared readily from tissues after oral intake ceases.

It is striking that so little toxicant is excreted in the feces. This indicates either destruction in the gut or more or less complete absorption. If the latter occurred one might expect larger quantities in the blood or urine unless, as the preliminary incubation studies indicate, the liver is particularly efficient in metabolizing the gamma isomer.

SUMMARY

1. Using the house fly as a sensitive test insect, a bio-assay method has been developed for the gamma isomer of benzene hexachloride.
2. Examination of the tissues of rats which had consumed gamma isomer in their diet has shown the presence of a substance having toxicologically identical characteristics to that of the gamma isomer.
3. Largest quantities of toxicant were found in fat and kidney tissues with measurable amounts in blood, liver, spleen, muscle, brain and adrenal.
4. Some excretion of toxicant occurs via the urine but little or none via the feces.

Acknowledgment. The author wishes to thank Mr. J. H. Fales, Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture, for so kindly furnishing the house flies used in this study.

REFERENCES

- (1) LAUG, E. P.: THIS JOURNAL, 86: 324, 1946.
- (2) BLISS, C. I.: Science, 69: 403, 1934.
- (3) LAUG, E. P., AND FITZHUGH, O. G.: THIS JOURNAL, 87: 18, 1946.
- (4) WOODARD, G., AND OFNER, R. R.: Fed. Proc., 5: 215, 1946.

PHARMACOLOGIC COMPARISON OF THE OPTICAL ISOMERS OF METHADON

C. C. SCOTT, E. B. ROBBINS AND K. K. CHEN

The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis 6, Indiana

Received for publication March 6, 1948

Our previous work (1, 2) on methadon was carried out with the racemic mixture. Doctors T. P. Carney and A. Pohland of our organic chemical division recently succeeded in resolving the product into its optical antipodes through the nitrile process, and afforded us an opportunity to ascertain the differences of pharmacological action between the two forms.

Published information from various laboratories on the relative activity and toxicity of the optical isomers of methadon is already on record. The Germans as interrogated by the British Intelligence Objectives Sub-Committee (3) apparently believed that the *l*-isomer was 4 times as active as the *d*-form. Thorp, Walton, and Ofner (4) attributed the analgesic activity to the *l*-isomer, since they observed no effect with the *d*-isomer. Eddy (5) and Luduena and his associates (6) arrived at a similar conclusion. Isbell and Eisenman (7) reported that in man the *l*-isomer was capable of reducing the intensity of morphine abstinence, while the *d*-isomer had no effect. Denton and his co-workers (8) recorded the same incidence of side reactions in human subjects with a dose of the racemic mixture twice that of the *l*-isomer.

The optically active isomers of Dolophine (methadon, Lilly) used in this investigation were in the form of hydrochlorides. They had the same melting points, 239–241°C., but opposite rotations—the specific rotation of the *l*-form being $[\alpha]_D^{23.5} - 128^\circ$, and that of the *d*-form, $[\alpha]_D^{24} + 126^\circ$, in 1 per cent aqueous solutions.

1. Analgesic potency—rats. By the tail-pinching technique of Haffner (9), the analgesic action of *d*- and *l*-Dolophine was measured in 50 albino rats. The drugs were injected intraperitoneally, and the duration and intensity of analgesia ascertained thereafter. Various dose levels were tried with both isomers until dosages of each which produced equal analgesic effects were determined. Thus, a dose of 4.0 mg. of *l*-Dolophine per kg. was found to equal 30 mg. of *d*-Dolophine per kg., which makes the *l*-isomer 7.5 times as active as the *d*-form in this species. Both compounds produced profound analgesia. The *l*-Dolophine also was found experimentally to be 1.6 times as potent in rats as racemic Dolophine, which checked well with the theoretical predicted value calculated from individual potencies mentioned above.

Dogs. Studies were made in 5 trained, unanesthetized dogs using the Andrews and Workman modification (10) of the Hardy-Wolff-Goodell procedure (11). As with rats, attempts were made to find doses of each compound which would be equal analgesically. Figure 1 shows some of the results. The *d*-isomer was still less potent in this species. In the dog, 1 mg. of *l*-Dolophine per kg. caused a rise

of the pain threshold greater than that produced by the *d*-compound in doses of 22.5 mg. per kg. and less than 30 mg. per kg. Roughly, the *l*-form was 25 times as potent as *d*-Dolophine. Both isomers were injected subcutaneously.

Man. In Figure 2 is seen the analgesic action of 160 mg. of *d*-Dolophine compared to that of only 3 mg. of the *l*-isomer, following oral administration. Pain thresholds were ascertained by the Hardy-Wolff-Goodell apparatus. These doses gave practically identical results. In the 3 subjects tested, the *l*-isomer was thus about 50 times as strong analgesically as the *d*-form.

This difference in relative potency in different species had not been encountered before in our studies of Dolophine, demerol, and related compounds. Previously

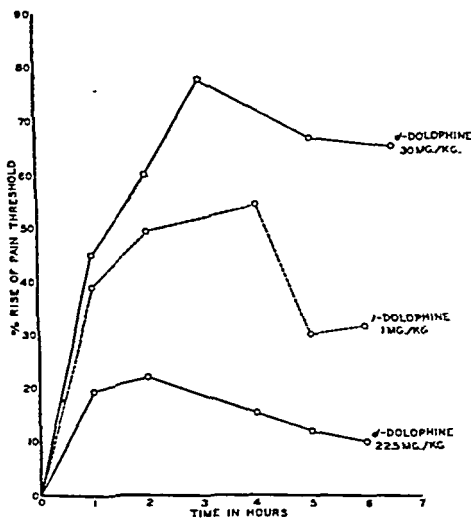


FIG. 1. ANALGESIC RESPONSES TO DOLOPHINE ISOMERS BY TRAINED UNANESTHETIZED DOGS

Each curve is an average for the same 4 animals.

(2), we had reported that the relative strength of any 2 substances was the same in rats, dogs, and men.

2. Signs and symptoms. In this connection, certain signs and symptoms were common to both isomers in man and dog. There were differences, however, which were quite striking. In human beings, doses of the 2 isomers which gave equivalent analgesic responses resulted in marked differences in the subjective symptoms. For example, in man a dose of 3 mg. of *l*-Dolophine, taken orally, caused definite lightheadedness, sleepiness, and apparently some interference with ability to think or concentrate. These symptoms persisted for 9 to 14 hours. On the other hand, 160 mg. of *d*-Dolophine by mouth produced either a feeling of well-being (euphoria ?) or a tendency toward lethargy with little or no lightheadedness. In the case of the *d*-isomer, the symptoms lasted only 4 to 5 hours.

Similar findings were noted in our trained dogs, although it was more difficult to judge the degree of side-effects. Also, equal analgesic responses to the 2 isomers were not obtained. Comparison of signs following the 30-mg.-per-kg. and 1-mg.-per-kg. doses, respectively, of *d*- and *l*-Dolophine is of interest. Although this dose of *d*-isomer caused marked analgesia, the narcotic effects appeared to be distinctly less than those resulting from *l*-Dolophine. In the animals which received the *l*-form, well-developed signs were noted, these changes being similar to those reported previously (1) for the racemic mixture. These signs tended to be slight or absent after *d*-Dolophine, and 1 dog had strong convulsive seizures.

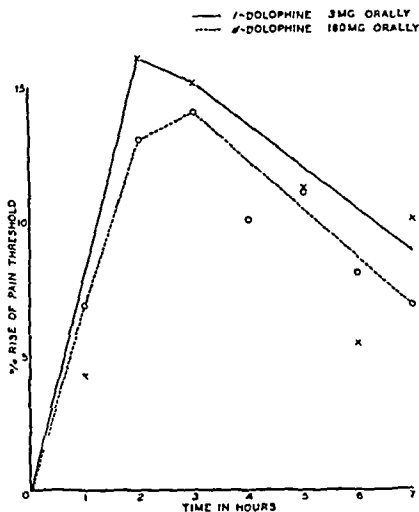


FIG. 2. EFFECT ON PAIN THRESHOLD IN MAN

The curves are averages for 2 subjects, the same individuals being used in each case.

3. *Effects in anesthetized dogs.* Experiments were made in 6 dogs under barbiturate anesthesia to determine whether or not there were differences in action of the 2 isomers on circulation, respiration, or intestinal motility. The effects on heart rate and blood pressure proved to be too slight or too irregular to distinguish the isomers. The *l*-compound, however, was approximately 25 times as potent a respiratory depressant as *d*-Dolophine, as judged by effect on respiratory minute volume. This figure agrees with the relative analgesic potency of the isomers in this species. On the other hand, 65 times as much *d*-Dolophine as the *l*-isomer was needed to produce a minimal increase in motility of the duodenum. The respiratory effect is probably related to narcotic action, while intestinal stimulation is parasympatheticomimetic.

4. *Acute toxicity.* The median lethal dose by intravenous administration into mice was determined simultaneously for *d*- and *l*-Dolophine as well as the racemic

mixture. Results are shown in table 1. Although there was no significant difference statistically between the optical isomers, the toxicity curves and objective signs were strikingly dissimilar. The toxicity curve for the *l*-compound was quite flat, deaths occurring over a period of a few minutes to as long as 8 hours after injection. These mice exhibited the usual signs of narcosis seen with racemic Dolophine. In contrast, death always occurred rapidly or not at all with the *d*-form, resulting in a steep toxicity curve. Violent convulsions preceded death. No period of narcosis was evident at any time.

An unexpected result was that *dl*-Dolophine was more toxic than either optical isomer. The difference was statistically significant. We repeated the toxicity test at another time with the same results. Also, we prepared a 50-50 mixture of the pure isomers and found that this mixture gave the same toxicity as the

TABLE 1

The median lethal doses of d-, l-, and dl-Dolophine by intravenous administration into albino mice

COMPOUND	DOSE	NUMBER DIED/NUMBER USED	LD ₅₀ ± S.E.
	mg. per kg.		mg. per kg.
<i>dl</i> -Dolophine	15	1/10	20.9 ± 1.6
	20	5/10	
	25	7/10	
<i>l</i> -Dolophine	15	2/10	28.7 ± 4.5
	20	1/10	
	25	5/10	
	30	7/10	
<i>d</i> -Dolophine	28	2/10	30.6 ± 1.0
	32	6/10	
	37	10/10	

regular Dolophine. Apparently, there is a synergistic action in toxicity between the isomers.

Both the *d*- and *l*-isomers were found to produce the Straub tail phenomenon in mice. The threshold doses were found to be about 2.0 and 0.5 mg. per kg. intravenously for *d*- and *l*-Dolophine, respectively. In this respect, then, the *l*-isomer appears to be 4 times as potent as *d*-Dolophine.

DISCUSSION. Our results show that the analgesic action of *l*-Dolophine is definitely stronger than the *d*-form; the ratio of activity varies according to species, being approximately 7.5:1 in rats, 25:1 in dogs, and 50:1 in man. *d*-Dolophine uniformly produces analgesia in relatively larger doses. In this respect, our data are at variance with those of Thorp, Walton, and Ofner (4). It is interesting to point out that in anesthetized dogs the *l*-isomer as compared with the *d*-isomer is 25 times as depressant on respiration, but 65 times as stimulating to the duodenum. Meanwhile, there is very little difference between the two on the heart rate and arterial blood pressure. In both dogs and man, a greater de-

pressant or narcotic action occurs with the *l*-isomer in doses which cause the same degree of analgesia as with equivalent doses of the *d*-isomer. It is difficult to offer adequate explanations for all the observations so far recorded.

Equally puzzling is the difference in toxicity between the *l*- and *d*-isomers on the one hand, and the *dl*-isomer on the other, the latter being more toxic than either of the other two. Our results are not in agreement with those of Jenney and Pfeiffer (12) and Hopper and Miller (13).

SUMMARY

1. The analgesic action of *l*-Dolophine is much greater than that of the *d*-isomer, the order of activity being approximately 7.5:1 in rats, 25:1 in dogs, and 50:1 in man.

2. *l*-Dolophine produces more narcosis than the *d*-form in equianalgesic doses.

3. In anesthetized dogs, *l*-Dolophine is 25 times as depressant to respiration, but 65 times as stimulating to duodenum, as the *d*-isomer.

4. There is little difference in toxicity between the *l*- and *d*-isomers in mice by intravenous injection. Either optical isomer is, however, slightly less toxic than the racemic mixture.

REFERENCES

1. SCOTT, C. C., AND CHEN, K. K. THIS JOURNAL, 87: 63, 1946.
2. SCOTT, C. C., KOHLSTAEDT, K. G., AND CHEN, K. K. Anesth & Analg, 26: 12, 1947.
3. HEPWORTH, H., BARBER, H. J., HELMS, B. A., KENNEDY, W. P., PARISH, H. J., THOMPSON, H. W., AND WHITE, A. C. Brit Intelligence Objectives Sub-Committee, Final Rep. No. 116, Item No. 24, p. 65, 1946.
4. THORP, R. H., WALTON, E., AND OFNER, P. Nature, 159: 679, 1947, 160: 605, 1947.
5. EDDY, N. B. Personal communication.
6. LUDUENA, F. P., MILLER, L. C., ANANENKO, E., AND FRICK, J. D. Fed. Proc., 7: 241, 1948.
7. ISBELL, H., AND EISENMAN, A. J. Fed. Proc., 7: 162, 1948.
8. DENTON, J. E., STRAUS, O. H., WADDELL, W. E., AND BELCHER, H. K. Fed. Proc., 7: 214, 1948.
9. HAFNER, F. Deutsche med. Wchnschr., 55: 731, 1929.
10. ANDREWS, H. L., AND WORKMAN, W. THIS JOURNAL, 73: 99, 1941.
11. HARDY, J. D., WOLFF, H. G., AND GOODELL, H. J. Clin. Investigation, 19: 649, 1940.
12. JENNEY, E. H., AND PFEIFFER, C. C. Fed. Proc., 7: 231, 1948.
13. HOPPER, J. O., AND MILLER, L. C. Fed. Proc., 7: 228, 1948.

THE RODENTICIDAL ACTIVITY OF FLUOROACETPHENYLHYDRAZIDE ("FANYLINE") AND ITS ORAL TOXICITY TO SEVERAL SPECIES

WITH A NOTE ON THE TOXICITY OF 64 OTHER CANDIDATE RODENTICIDES

LEONARD KAREL*

WITH THE TECHNICAL ASSISTANCE OF MRS. J. L. HARRISON

*Toxicology Section, Medical Division, Army Chemical Center, Maryland**

Received for publication March 8, 1948

Following oral toxicity screening tests with 65 compounds¹ as a preliminary to the determination of their rodenticidal potentialities, it was found that the phenylhydrazine derivative of fluoroacetic acid ("Fanyline") was the only compound of the entire series with properties which warranted its further investigation as a possible rodenticide.

Earlier studies directed towards the synthesis of effective verminicides led to the discovery of sodium fluoroacetate ("1080"), the toxicity and pharmacology of which have been reported by Kalmbach (1), Dieke and Richter (2), Chenoweth and Gilman (3), and Ward and Spencer (4).

Kalmbach (1) gave the approximate median lethal doses of "1080" when administered in food baits as 5.0 mg./kg. for deer mice, the wood rat, and wild Norway rats; 2.5 mg./kg. for tame white rats; 0.5 mg./kg. for meadow mice; and 0.1 mg./kg. for wild black rats. He reported also that laboratory rats ingesting sublethal doses for 5 to 14 days acquire a tolerance which may last 7 days. Dieke and Richter (2), on the other hand, stated that when "1080" was administered in 10 per cent acacia by stomach tube to wild Norway rats fasted overnight, the $LD_{50} \pm S.E.$ in mg./kg. was only 0.22 ± 0.01 , the survival time varying from 45 to 240 minutes.

Data on the toxicology, pharmacology, and pathology of phenylhydrazine may be found in the papers by von Oettingen and Deichmann-Gruebler (5), von Oettingen *et al* (6), and Hueper (7). In the former (5), it is stated that a subcutaneous dose of 0.18 gm./kg. produced 100 per cent fatalities within 50 minutes in mice, whereas a dose of 0.17 gm./kg. killed only 45 per cent of the mice in 70 minutes. The "minimal fatal" dose for rats by subcutaneous injection is given as also 0.18 gm./kg. by von Oettingen *et al* (6). No information, however, was found in the literature regarding the biological assessment of the phenylhydrazine derivative of fluoroacetic acid.

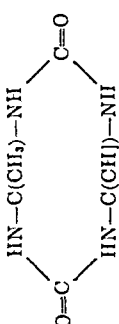
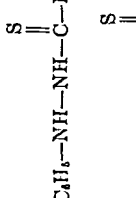
Since "Fanyline" is only slightly water soluble, whereas one of the drawbacks of "1080" is its marked water solubility, it seemed that for this reason, in addi-

* Present Address—Division of Research Grants and Fellowships, the National Institute of Health, Bethesda 14, Maryland

¹ These compounds were submitted by Doctor Benjamin Witten of the Army Chemical Corps, Technical Command, Army Chemical Center, Maryland.

TABLE I
The oral toxicity of 68* candidate rodenticides to non-fasted wistar, albino rats weighing 200-500 gm. and observed for 7 days

NO.	NAME OF COMPD.	FORMULA	MOL. WT.	SEX	MORTALITY FRACTION AT A DOSE OF MG./KG.	
					100	50
1	β -chlorovinylarsonic Acid	$\text{Cl}-\text{CH}=\text{CHAs}=\text{O} \cdot (\text{OH})_2$	186.42	M	3/10	1/10
2	Diphenylarsinic Acid Nitrate	$(\text{C}_6\text{H}_5)_2\text{As}-\text{OH} \cdot \text{HNO}_3$	309.24	F	9/10	5/9
3	Phenylarsonic Acid	$\text{C}_6\text{H}_5-\text{As}=\text{O} \cdot (\text{OH})_2$	202.03	F	7/10	1/10
4	Methyl-di-(β -chloroethyl) ammonium picrate	$(\text{Cl}-\text{CH}_2-\text{CH}_2)_2\text{N}-\text{CH}_2-\text{OH}-\text{C}_6\text{H}_4(\text{NO}_2)_3$	384.16	M	3/7	6/8
5	Tri-(β -chloroethyl) ammonium picrate	$(\text{Cl}-\text{CH}_2-\text{CH}_2)_3\text{N}-\text{OH}-\text{C}_6\text{H}_4(\text{NO}_2)_3$	433.64	M	4/10	4/10
6	Ethyl-di-(β -chloroethyl) ammonium picrate	$(\text{Cl}-\text{CH}_2-\text{CH}_2)_2\text{N}-\text{C}_2\text{H}_5-\text{OH}-\text{C}_6\text{H}_4(\text{NO}_2)_3$	399.19	M	3/10	2/10
7	Di-(β -chloroethyl) sulfinop-toluenesulfonylimine	$(\text{CH}_2-\text{CH}_2-\text{Cl})_2-\text{S}=\text{N}-\text{SO}_2-\text{C}_6\text{H}_4-\text{CH}_3$	328.27	F	1/11	2/11
8	Bis-[β -(β -naphthoxy) ethyl] sulfide	$(\text{C}_{10}\text{H}_7-\text{O}-\text{CH}_2-\text{CH}_2)_2\text{S}$	374.48	F	1/10	0/10
9	1,2-Bis-[β -(β -naphthoxy) ethylthio] ethane	$(\text{C}_{10}\text{H}_7-\text{O}-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_2)_2$	434.59	M	0/10	1/10
10	Ethyl 2,4,6-tribromophenoxyacetate	$\text{C}_6\text{H}_3\text{Br}_3\text{O}-\text{CH}_2\text{COOC}_2\text{H}_5$	416.90	F	1/10	1/10
11	7,8-Diphenylglucuril	$\begin{array}{c} \text{NH}-\text{C}(\text{C}_6\text{H}_5)_2-\text{NH} \\ \diagup \quad \diagdown \\ \text{O}=\text{C} \quad \text{C}=\text{O} \\ \diagdown \quad \diagup \\ \text{NH}-\text{C}(\text{C}_6\text{H}_5)_2-\text{NH} \end{array}$	294.30	M	1/10	1/10
12	2,2',4,4',6,6'-Hexachloro-N,N'-diphenylurea	$(\text{C}_6\text{H}_2-\text{Cl}_3-\text{NH})_2\text{CO}$	418.94	F	0/10	1/10
13	Methyl-bis-[β -(phthalimido) ethyl] amine	$[\text{C}_6\text{H}_4(\text{CO})_2\text{N}-\text{CH}_2-\text{CH}_2]_2-\text{NCH}_3$	377.38	M	1/10	0/10

14	1,2-Bis-(β -phthalimido) ethylthio] ethane	$[\text{C}_6\text{H}_4(\text{CO})_2\text{N}-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_2]_2$	140.52	M	1/10	0/8
15	N'-methyl-N'-phenyl thiourea	$\text{C}_6\text{H}_5\text{NHSC}-\text{NHCH}_3$	166.24	F	0/10	1/10
16	Methylarsylene-bis-(N-pentamethylenedithiocarbamate)	$\text{CH}_3-\text{As}-(\text{S}-\text{C}=\text{S}(\text{C}_4\text{H}_9\text{N}))_2$	407.48	F	0/10	2/10
17	Fluoroacetphenylhydrazide	$\text{C}_6\text{H}_5\text{NH}-\text{NHCOCH}_2\text{F}$	168.17	F	10/10	7/7
18	β Fluoroethyl, N-(α -Naphthyl) carbamate	$\text{C}_{10}\text{H}_7\text{NHCOOCH}_2\text{CH}_2\text{F}$	233.23	M	7/10	3/10
19	Bis [di-(β -chloroethyl) sulfide] Palladium chloride	$(\text{Cl}-\text{CH}_2\text{CH}_2)_2\text{S}_2\text{PdCl}_2$	195.77	F	1/10	1/10
20	Bis-[β -(p-phenylazo-phenoxy) ethyl] sulfide	$(\text{C}_6\text{H}_5\text{N}=\text{N}-\text{C}_6\text{H}_4\text{OCH}_2\text{CH}_2)_2\text{S}$	151.58	M	1/10	0/10
21	ω -chloroacetophenone-2,4-dinitrophenylhydrazone	$\text{C}_6\text{H}_5\text{C}(\text{CH}_2\text{Cl})=\text{N}-\text{NH}-\text{C}_6\text{H}_3(\text{NO}_2)_2$	334.72	M	1/10	0/10
22	Bis-(tri-(β -chlorovinyl)arsine] palladium chloride	$[(\text{Cl}-\text{CH}=\text{CH})_2\text{As}]_2\text{PdCl}_2$	696.39	F	1/10	0/10
23	7,8-Dimethylglycoluril		170.17	M	2/10	3/10
24	Methyl-bis-(β -p-(phenylazo-phenoxy) ethylamine	$(\text{C}_6\text{H}_5\text{N}=\text{N}-\text{C}_6\text{H}_4\text{O}-\text{CH}_2\text{CH}_2)_2\text{NCH}_3$	479.56	F	2/10	0/5
25	Tris [β -(p-phenylazo phenoxy) ethyl] amine	$(\text{C}_6\text{H}_5\text{N}=\text{NC}_6\text{H}_4\text{O}-\text{CH}_2-\text{CH}_2)_3\text{N}$	689.79	M	0/10	1/10
26	Phenylthiosemicarbazide		167.23	M	1/4	1/4

tion to its toxicity, further investigation of this product was indicated. Inasmuch as wild rats and mice were not available, all toxicity and acceptability tests were conducted with Wistar albino rats and Carworth Farms albino mice. Supplementary oral toxicity data were determined in several other laboratory species.

EXPERIMENTAL

All compounds screened for their oral toxicity to rats were administered through #14, 2½ inch hypodermic needles with ends blunted and protected with sealing wax. Non-water soluble compounds were suspended in 20 per cent acacia and given as such.

Animals were divided into two groups regardless of weight, no rats, however, weighing less than 200 nor more than 500 grams. Each of these groups, except

TABLE 2

Oral toxicity of fluoroacetphenylhydrazide for rats, mice, pigeons, rabbits, guinea pigs, cats, and dogs

SPECIES (AND NO. OF ANIMALS USED IN THE ASSAY)	LD ₅₀ ± STD ERROR	SLOPE OF REGRESSION LINE-b	HIGHEST DOSE PRODUCING 0 PER CENT MORTALITY	LOWEST DOSE PRODUCING 100 PER CENT MORTALITY
	mg/kg		mg/kg	mg/kg
Rats (80)	9.1±0.7	4.5	2.5	16.1
Mice (100)	44.9±2.9	7.0	10.0	70.0
Pigeons (37)	7.2±0.6	8.5	5.8*	12.5
Rabbits (18)	—	—	1.3†	1.7
Guinea Pigs (28)	—	—	0.65	1.3
Cats (12)	—	—	0.25	0.5
Dogs (12)	—	—	0.1	0.25

* Mortality = 3/12.

† Mortality = 3/6.

as otherwise noted in table 1, consisted of 10 rats, one series of 10 receiving 100 mg./kg. of body weight and the other 50 mg. of a compound per kg. body weight. Solutions and suspensions were made to contain 25 mg. of agent per ml. of vehicle so that the volume given was no greater than 1 ml./250 gm. of body weight. Test animals were observed several times daily for a period of 7 days.

Since no compounds the oral toxicity of which was such as to permit several survivals at the 50 mg. level were further investigated, median lethal dose studies were conducted only with "Fanyline," which had killed all rats at both the 100 and 50 mg. dosages. The data for the entire series of compounds screened appear in table 1.

The method used to determine the oral LD₅₀ of "Fanyline" in both rats and mice was similar to that used in the screening. Suspensions were adjusted so that the volume of the total dose was contained in 0.05 ml. for each 20 gm. of body weight for mice. The median lethal doses and their standard errors, calculated by the method of Bliss (8), are in table 2.

It was found that for male rats weighing between 200 and 350 gm., the median lethal dose was 9.1 mg./kg., the standard error being 0.7 mg./kg., while for female mice between 17 and 23 gm., the corresponding values were 44.9 ± 2.9 mg./kg.

TABLE 3

Acceptance and lethality of 1% and 2% mixtures of fluoroacetphenylhydrazide in corn meal to non pre-baited rats and mice

SPECIES AND SEX	NO. OF ANIMALS IN EACH GROUP	WT. RANGE	POISON CONSUMED	STRENGTH OF MIXTURE	MORTALITY	LENGTH OF EXPOSURE TO BAIT	COMMENT
		gm	mg/kg/animal	per cent		hrs.	
Rat-M	1	500	74	2	1/1	.1	Fasted 48 hrs.
" -M & F	9	205-419	Not recorded	2	9/9	2.5	Fasted 48 hrs.
" -M	1	323	74	2	1/1	24	Non-fasted
" -M & F	7†	ca.300-500	Not recorded	2	7/7	48	Choice of poisoned meal and fox chow, Non-fasted
" -M & F	4†	320-355	42-104	1	4/4	24	Non-fasted
" -M & F	5†	240-360	16-83	1	5/5	3.25	" "
" -M & F	5†	230-325	62-138	1	5/5	2	" "
Rat-F	3	ca. 76	Not recorded	1	3/3	24	Non-fasted
" -F	3	65-75	" "	1	3/3	24	" "
" -M	3	65-82	" "	1	3/3	24	" "
" -F	3	65-82	" "	1	3/3	24	" "
" -F	4	71-79	" "	1	4/4	24	" "
Mice-M & F	25	26-30	104 for all mice	2	12/25†	4	Non-fasted
" -M & F	25	26-30	74 " " "	2	9/25†	4	Non-fasted
" -M & F	25	26-30	84 " " "	2	10/25†	4	" "
" -M & F	25	26-30	114 " " "	2	2/25†	4	" "

* Deaths generally occurred within 24 hours from time of exposure in rats and somewhat more slowly in mice.

† One animal per cage.

‡ Survivors were pre-baited for one week after which they were exposed to contaminated bait. The following deaths occurred 13/13, 13/16, 8/15, and 21/23, respectively.

Acceptability tests were conducted, without prebaiting, with 1 and with 2 per cent of the "Fanyline" in corn meal. In some tests, rats were fasted overnight before being placed in the presence of contaminated corn meal. In others, the animals were not fasted. Finally, well-fed, non-starved rats not previously exposed to either contaminated or uncontaminated corn meal were allowed to choose between contaminated meal and the usual block chow. In all instances, even when the choice was allowed, the animals ate freely of the poisoned corn meal. Quantitative records were kept in a number of these experiments.

In the case of the mice, individual quantitative food consumption data were not obtained because of non-availability of suitable feeding cages.

The data on the feeding experiments may be found in table 3.

Following the tests which indicated that the fluoroacetphenylhydrazide was readily accepted by rats, toxicological investigations were conducted on pigeons, rabbits, guinea pigs, cats, and dogs. In the three former species, the compound suspended in 20% gum acacia was administered orally by tubing with a stiff rubber catheter, while in the two latter, the "Fanyline" was given in capsules. The acacia suspensions were adjusted to contain the required dose in 1 ml./250 gm. body weight in pigeons and guinea pigs and in 1 ml./kg. in rabbits.

The calculated median lethal dose plus or minus one standard error was 7.2 ± 0.6 mg./kg. for pigeons; whereas the estimated median lethal doses were slightly less than 1.3 mg./kg. in rabbits, between 0.65 and 1.0 in guinea pigs, greater than 0.25 and less than 0.5 in cats, and between 0.1 and 0.25 in dogs. More complete data may be found in table 2.

The symptomatology resulting from the acute poisoning was similar to that previously reported (3). In rats and in mice, occasional delayed deaths were observed.

DISCUSSION

A survey of the species toxicities of the several fluoroacetates and "Fanyline" affords the belief that the primary action of the latter is due to the fluoroacetate moiety of the molecule. While further investigations with the phenylhydrazide would be of interest, such are not warranted unless the results of trials being currently conducted elsewhere on wild rats and other pests recommend the field use of the derivative.

SUMMARY

1. Fluoroacetphenylhydrazide, "Fanyline," a potential rodenticide, has been found to be readily accepted by laboratory Wistar rats and C. F. mice.
2. The oral median lethal dose (± 1 std. error) of the compound was 9.1 (± 2.7) mg./kg. for rats, 44.9 (± 2.9) for mice, and 7.2 (± 0.6) for pigeons. For rabbits, the LD_{50} is probably slightly less than 1.3 mg./kg.; for guinea pigs, between 0.65 and 1.3; for cats, greater than 0.25 and less than 0.5; and for dogs, between 0.1 and 0.25 mg./kg.

REFERENCES

- (1) KALMBACH, E. R.: Science 102: 232, 1945.
- (2) DIEKE, S. H., AND RICHTER, C. P.: Pub. Health Reports 61: 672, 1946.
- (3) CHENOWETH, M. B., AND GILMAN, A.: THIS JOURNAL, 87: 90, 1946.
- (4) WARD, J. C., AND SPENCER, D. A.: J. Am. Pharm. Assoc., Sci. Ed. 36: 59, 1947.
- (5) VON OETTINGEN, W. F., DEICHMANN-GRUEBLER, W., AND HUEFER, W. C.: J. Ind. Hyg. & Tox., 18: 301, 1936.
- (6) VON OETTINGEN, W. F., AND DEICHMANN-GRUEBLER, W.: J. Ind. Hyg. & Tox. 18: 1, 1936.
- (7) HUEFER, W. C.: J. Ind. Hyg. & Tox. 18: 17, 1936.
- (8) BLIES, C. I.: Quart. J. Pharm. Pharmacol. 11: 192, 1938.

SOME ASPECTS OF THIAMINE TOXICITY¹

JAY A. SMITH, PIERO P. FOA, HARRIET R. WEINSTEIN,
A. S. LUDWIG, AND J. MARVIN WERTHEIM

The Chicago Medical School, Chicago 12, Illinois

Received for publication March 11, 1948

During the course of other studies (1), it was noted that thiamine hydrochloride was toxic to dogs when injected intravenously in large doses. The literature reveals that toxic manifestations have been observed by many who gave thiamine in doses far greater than those at which thiamine acts as a vitamin. For example, repeated administrations caused hyperventilation, allergic reactions and even death (2-9); the intravenous, intramuscular or intraspinal injection of large doses of thiamine produced respiratory failure, decrease in blood pressure, vasodilation, and depression of the central nervous system (10-13); direct application of thiamine solutions to the cerebral cortex caused localized convulsions (14). These papers establish the fact that thiamine can be toxic. The present study was undertaken to extend the knowledge of the toxicity of thiamine and to investigate the mechanism of its toxic actions.

EXPERIMENTAL

Our results may be divided, for convenience, into three divisions; the effects of thiamine on the intact animal, the effects of thiamine on the vascular system, and the effects of thiamine on the heart.

The effects of thiamine on the intact animal

MATERIALS AND METHODS. Thiamine hydrochloride², in a freshly prepared 5% solution in saline, either acidic or neutralized immediately before use, was given in single doses from 10 to 500 mgm./kgm. into the femoral vein of dogs, under ether anesthesia. Artificial respiration was provided when desired. Blood pressure was recorded from the carotid artery with a mercury manometer. Respiration was recorded either by pneumographs or from a side arm of the tracheal cannula. The electrocardiogram was recorded from Lead II or from the three classical leads.

Blood samples were analyzed for total thiamine by the method of Hennessey and Cerecedo (15), slightly modified (1). The hydrogen ion concentration of blood was measured by withdrawing blood from the femoral vein into a shell vial, stoppering with minimum contact with air, and measuring with a Beckman pH-meter after the samples had come to room temperature. The dogs were heparinized to prevent coagulation.³

RESULTS. On injecting thiamine, 50 mgm. per kgm. intravenously into dogs, respiration is inhibited or ceases, arterial blood pressure decreases and an inverted T-wave frequently appears in the electrocardiogram. (Fig. 1.)

¹ This investigation was made possible by grants from the John and Mary R. Markle Foundation and from the Upjohn Company, Kalamazoo, Michigan.

² Thiamine hydrochloride was furnished by the Upjohn Company, Kalamazoo, Michigan.

³ Heparin was kindly supplied to us by the Abbott Laboratories, North Chicago, Illinois, and Wilson and Company, Inc., Chicago, Illinois.

Fig. 1A also shows that if artificial respiration is maintained, blood pressure returns to normal, the abnormal T-wave disappears, and, eventually, spontaneous respiration is resumed. On the other hand, (Fig. 1B) if artificial respiration is not provided, the animal dies from respiratory arrest, accompanied by changes in blood pressure and the electrocardiogram typical of anoxia.

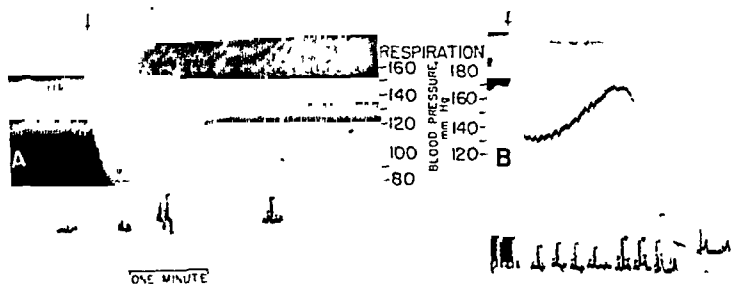


FIG. 1. The effect of thiamine on the respiration, blood pressure and electrocardiogram of dogs. Thiamine hydrochloride, 59 mgm/kgm, was injected at each arrow. Electrocardiograms from Lead II A With artificial respiration. Larger excursions at slower rate are due to artificial respiration. Smaller excursions at faster rate are due to natural respiration B. Without artificial respiration

TABLE 1

The changes in hydrogen ion concentration (pH) of blood after the intravenous injection of thiamine (50 mgm/kgm)

TIME, SECONDS AFTER THIAMINE	ACID THIAMINE, MINIMUM ARTIFICIAL RESPIRATION (3 EXPERI- MENTS)	ACID THIAMINE, HYPERVEN- TILATION (5 EXPERI- MENTS)	ACID THIAMINE, NO ARTIFICIAL RESPIRATION (3 EXPERI- MENTS)	ALKALINE THIAMINE, MINIMUM ARTIFICIAL RESPIRATION (3 EXPERI- MENTS)	ALKALINE THIAMINE, HYPERVEN- TILATION (4 EXPERI- MENTS)	ALKALINE THIAMINE NO ARTIFICIAL RESPIRATION (2 EXPERI- MENTS)
	pH	pH	pH	pH	pH	pH
0	7.59	7.52	7.52	7.61	7.70	7.63
10	7.56	7.53	7.47	7.67	7.68	7.60
20	7.57	7.57	7.44	7.62	7.64	7.58
30	7.53	7.58	7.44	7.63	7.65	7.56
40	7.53	7.64	7.50	7.62	7.65	7.56
50	7.53	7.60	7.45	7.62	7.66	7.51
60	7.55	7.62	7.46	7.61	7.66	7.52
75-120	7.56	7.62	7.50	7.59	7.66	7.51
150-240	7.56	7.65	7.47 (Death)	7.58	7.66	7.41 (Death)

When thiamine hydrochloride is dissolved in water, it produces a solution whose acidity is proportional to the concentration. It was necessary, therefore, to investigate the effects of acidic and alkaline solutions of thiamine to clarify the effects of the acidity itself. It was found that thiamine solutions and neutral thiamine solutions produce identical effects on the intact dog.

Table 1 shows the effect of pH on blood after injections of thiamine into in-

tact dogs. It is seen that thiamine solutions, either acidic or neutralized, do not alter the pH of blood appreciably. The changes in pH that do occur are due to changes in respiratory ventilation and may be altered at will.

Table 2 demonstrates the effectiveness of artificial respiration in preserving the animals even after large doses of thiamine. It is seen that injections producing concentrations of less than about seven milligrams of thiamine per 100 cc. blood are not fatal without artificial respiration, doses producing greater

TABLE 2

The relation between thiamine injected and the resulting concentration in blood and the effect of artificial respiration on survival. Blood samples were taken 15-30 seconds after thiamine was injected. Results from 6 dogs

THIAMINE INJECTED	RESULTING CONCENTRATION OF THIAMINE IN BLOOD, MG. PER 100 CC.	ARTIFICIAL RESPIRATION	FATE
mgm./kgm.			
50.0	19.3	With	Recovery
34.5	7.8	Without	Death
50.0	21.4	With	Recovery
25.0	8.3	Without	Death
107.0	36.9	With	Recovery
17.9	10.0	Without	Death
125.0	20.6	With	Recovery
50.0	7.3	Without	Death
50.0	6.3	With	Recovery
16.7	6.9	Without	Recovery
32.4	7.8	Without	Recovery*
220.0	43.7	With	Recovery
220.0	119.5†	With	Recovery

* When it became evident that death was imminent, artificial respiration was started, and the dog recovered.

† This high value resulted from the injection of several previous doses of thiamine.

concentrations are fatal without artificial respiration, and doses producing concentrations up to one hundred and twenty milligrams thiamine per 100 cc. blood are tolerated if artificial respiration is maintained. In other experiments, much higher concentrations were not fatal if artificial respiration was provided.

In many experiments spontaneous respiration was resumed if artificial respiration was maintained for sufficiently long periods. Table 3 shows that after a large dose of thiamine, the concentration in the blood decreases rapidly; at the end of 2 hours, thirty-eight per cent of the thiamine injected was recovered in the urine. Thus urinary excretion is of great importance in eliminating thiamine from the blood; Table 3 also shows that even in the absence of functional

kidneys, as in the dog heart-lung preparation, the thiamine concentration in the blood decreases about eighteen per cent in fifteen minutes. Diffusion of thiamine from the blood into the tissues undoubtedly accounts for much of the decrease. From this table, it is apparent that thiamine concentration of blood decreases rapidly to tolerable levels, when spontaneous respiration may be resumed.

Table 4 shows the relation between blood pressure and the heart rate in the intact dog after thiamine. One can see that the fall in blood pressure produced

TABLE 3

The rate of disappearance of thiamine from the blood

TIME, MINUTES AFTER THIAMINE	A. INTACT DOG THIAMINE HYDROCHLORIDE, 100 MG/M /KGM, INJECTED INTRAVENOUSLY INTO A 10 KGM DOG URINE COLLECTED FROM CANNULATED URETERS			B. DOG HEART-LUNG PREPARATION THIAMINE HYDROCHLORIDE, 2000 MG/M, POURED INTO VENOUS RESERVOIR	
	Blood thiamine, mgm per 100 cc	Urinary thiamine		Concentration of thiamine, mgm /100 cc whole blood	
		Mgm excreted per minute	Total excreted, mgm (cumulative)		
0	0.002	—	—	0.00	
1	38.1	13.6	68.0	—	
2	19.7			740.0	
3	29.4			—	
4	12.2			—	
5	19.4			—	
7	11.7	13.2	133.6	—	
9	13.3			615.0	
11	13.1			—	
13	9.3	6.1	164.0	—	
15	9.8			583.0	
20	—	4.3	229.4	—	
30	—	2.8	271.7	—	
60	—	1.2	316.8	—	
120	—	0.7	381.0	—	

by thiamine is accompanied by a decrease in heart rate which persists even after the blood pressure has returned to normal.

Our next experiments were undertaken to investigate the mechanisms by which thiamine causes the observed fall in blood pressure. To this end, we investigated the effect of thiamine on the vascular system and on the isolated heart.

The effect of thiamine on the vascular system

Rates of perfusion through the isolated rabbit ear and through the femoral and mesenteric artery of the dog were measured.

MATERIALS AND METHOD. Perfusion of the rabbit ear was accomplished as follows: under anesthesia, the ear was excised, and the central artery was cannulated and perfused at constant pressure with mammalian Ringer with or without thiamine. The rate of

perfusion was expressed in terms of drops per minute. The hydrogen ion concentration of the perfusate was measured with the Beckman pH-meter before and after passing through the ear.

Rates of perfusing femoral and mesenteric arteries were measured as follows: a large branch of the femoral or mesenteric artery of the dog was isolated and cannulated. This was perfused with approximately four milliliters of saline solution (the actual amount was constant for each experiment) from a glass tube located about four feet above the dog; the time required for each perfusion was recorded by key and signal magnet on a kymograph. In this fashion, perfusion times were ascertained at half minute intervals until constant, thiamine was injected between two perfusion determinations, and the perfusion

TABLE 4

The effect of thiamine (50 mgm./kgm.) on blood pressure and heart rate. Rate was calculated from electrocardiograms. Results from 9 dogs.

	BEFORE THIAMINE INJECTION		AT TIME OF MINIMUM BLOOD PRESSURE AFTER INJECTION OF THIAMINE		AT TIME WHEN NATURAL RESPIRATION BEGAN AFTER INHIBITION BY THIAMINE	
	Blood pressure, mm. Hg	Rate, beats per minute	Change in blood pressure, mm. Hg, from preinjection level	Change in rate, beats per minute, from preinjection level	Change in blood pressure, mm. Hg, from preinjection level	Change in rate, beats per minute, from preinjection level
	118	200.0	-40	-27.6	+15	-34.3
	130	157.1	-50	-22.6	0	-20.4
	128	148.5	-48	-14.0	+4	-9.6
	134	139.5	-30	-2.7	+18	-3.1
	142	142.9	-48	+5.3	+4	-4.0
	98	214.2	-18	-39.8	-2	-58.8
	162	164.8	-19	-7.8	+13	+15.9
	120	188.6	-15	+57.3	+8	-17.2
	122	211.2	-28	-26.1	+23	-41.8
	146*	192.3	-34	-13.8	+4	-21.9
	126	221.5	-32	-18.4	+14	0
	120	194.4	-66	-27.8	-36	-18.2
Mean ..	128.8	181.3	-35.6	-11.5	+5.4	-17.8

* 100 mgm./kgm.

times were recorded again at half minute intervals for 10 minutes. Changes in the perfusion rate did not always follow changes in the arterial pressure, indicating that perfusion rates were not due exclusively to changes in the hydrostatic pressure of the collateral circulation. The perfused area of the leg or small intestine was invariably well supplied by collateral circulation, since blood flowed back into the cannula between perfusions.

RESULTS. The results of these experiments are presented in Table 5 and Fig. 2.

Table 5 shows that the rate of perfusion, in drops per minute, through the isolated rabbit ear increases with thiamine hydrochloride, and that neutralized thiamine solutions are without marked vasodilating effect. It must be concluded that vasodilation due to thiamine hydrochloride on the isolated blood vessels is due, essentially, to the acidity of the solutions.

Fig. 2 shows changes in perfusion rates (in terms of seconds required for perfusion of a constant volume of saline) of the femoral and mesenteric arteries and changes in blood pressure after injections of thiamine, 50 mgm./kgm., into dogs. The figure shows that a decreased perfusion time (i.e., an increased rate) accompanies the fall in blood pressure.

These results indicate that thiamine, in the isolated rabbit ear, may produce vasodilation, due principally to the acidity of the solutions. However, in the

TABLE 5

The effect of thiamine and hydrogen ion concentration on the rate of perfusion through the rabbit ear. Hydrogen ion concentrations were measured as the fluid entered and exited from the ear. Mean values from 18 experiments

SOLUTION	HYDROGEN ION CONCENTRATION		DROPS PER MINUTE	PER CENT CHANGE, drops per minute; (Thiamine-control) $\times 100$ Control
	Entering ear	Exiting from ear		
Ringer	pH 7.08	pH 7.55	14.0	+0.7
0.025% Thiamine (neutralized)	7.03	7.52	14.1	
Ringer	7.10	7.61	12.0	+15.0
0.025% Thiamine Hydrochloride	6.70	7.37	13.8	
Ringer	7.22	7.55	27.6	+0.4
0.05% Thiamine (neutralized)	7.25	7.50	27.7	
Ringer	7.23	7.50	15.6	+26.3
0.05% Thiamine Hydrochloride	6.37	6.91	19.7	
Ringer	7.13	7.55	35.2	-8.2
0.10% Thiamine (neutralized)	7.11	7.41	32.3	
Ringer	7.17	7.38	16.9	+18.9
0.10% Thiamine Hydrochloride	5.69	5.92	20.1	
Ringer	7.29	7.56	25.2	+0.8
0.20% Thiamine (neutralized)	7.10	7.38	25.4	
Ringer	7.16	7.40	26.4	+24.6
0.20% Thiamine Hydrochloride	4.92	5.02	32.9	

intact dog, even in the absence of pH changes in the blood, vasodilation occurs. We interpret these results to mean that vasodilation due to thiamine is not necessarily a direct peripheral action but is due to thiamine acting elsewhere, possibly on the central nervous system.

The effects of thiamine on the isolated heart

The isolated turtle heart and the dog heart-lung preparation were utilized in these studies.

MATERIALS AND METHODS. The turtle heart was isolated (16) and perfused at constant pressure with amphibian Ringer without or with thiamine. Contractions of the auricle and ventricle were recorded. The hydrogen ion concentration of the perfusate was measured with the Beckman pH-meter.

The classical dog heart-lung preparation was modified by employing five parallel rubber tubes in place of the usual arterial resistance; the tubes were partially closed with screw clamps to obtain maximum arterial pressure with maximum cardiac output. Thiamine

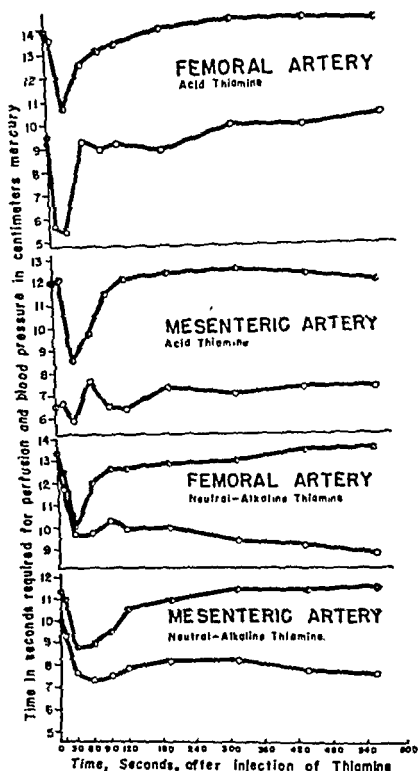


FIG. 2. The effect of thiamine, 50 mgm./kgm., on blood pressure and perfusion rate through the femoral and mesenteric arteries of the dog. Results of 8 experiments on 6 dogs; 4 additional dogs gave comparable results not included in these figures. ○ Perfusion times; ● arterial blood pressure.

hydrochloride, as a dry powder, was dumped into the venous reservoir where it dissolved immediately. Arterial and venous pressures were recorded with a mercury and water manometer respectively. Cardiac output was measured with a device located between arterial resistance and venous reservoir. The device consisted of a metal trough partitioned in the middle, with the center of gravity beneath the partition. One side filled with blood, tipping the device, tripping an electric contact connected to a signal magnet, and placing the other side in position to be filled. The device was carefully calibrated by measuring the total volume of blood passed when each bucket was filled and emptied ten times.

RESULTS. The results of experiments on isolated heart preparations are shown in Fig. 3 and Table 6.

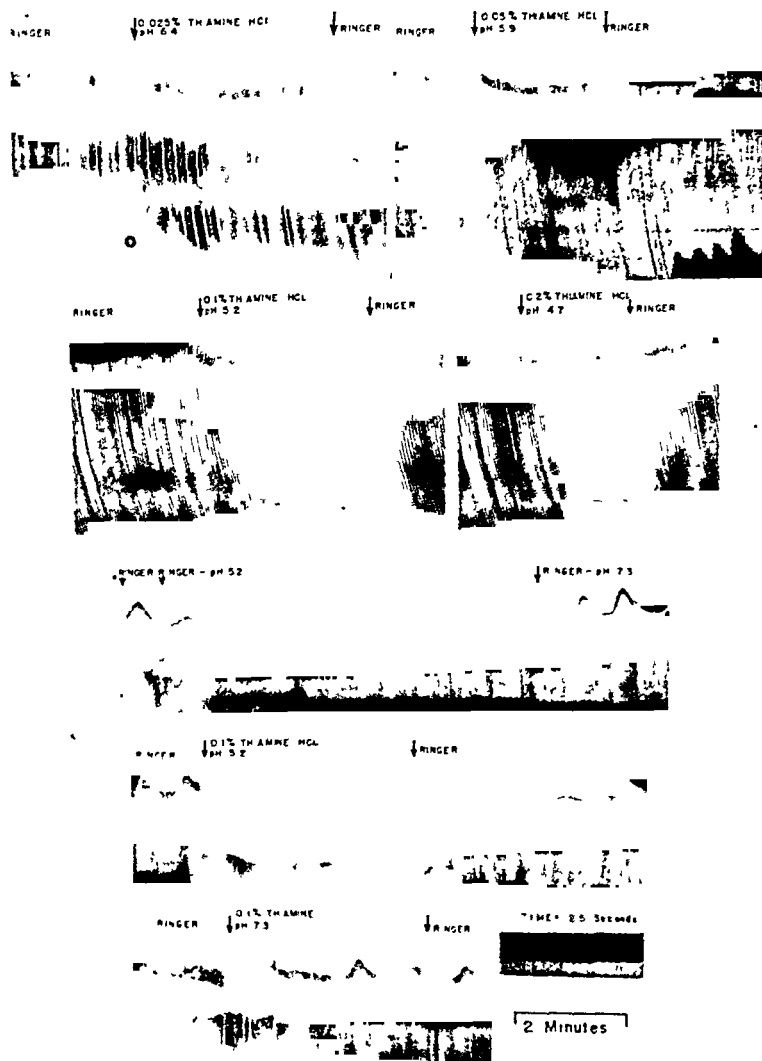


FIG 3 The effect of thiamine and hydrogen ion concentration on the turtle heart. Upper tracing, auricular contractions, lower tracings, ventricular contractions

Fig. 3 shows that the depression of the perfused turtle heart by thiamine hydrochloride solutions increases with an increase in concentration and an increase in acidity. Results obtained with neutralized thiamine and acidified Ringer

solutions indicate, however, that the depressant action resides principally in the acidity of the solutions.

Table 6 shows results obtained with the dog heart-lung preparation. It is apparent that concentrations of thiamine in the blood similar to those which cause a decrease in blood pressure and respiratory paralysis (Table 2) in the intact dog, produce no measurable changes in arterial pressure or cardiac output in the dog heart-lung preparation. However, when much higher concentrations are reached, arterial pressure and cardiac output decrease in rough proportion to the concentration of thiamine and the acidity of the blood. Venous pressure was not consistently raised or lowered by thiamine. Thus, in the dog

TABLE 6

The effect of thiamine hydrochloride on the dog heart-lung preparation. Summary of 30 observations on 5 successful preparations

DOSE THIAMINE, MGM.	RESULTING CONCENTRA- TION, MGM. THIAMINE PER 100 CC. BLOOD	HYDROGEN ION CON- CENTRA- TION OF BLOOD	ARTERIAL PRESSURE, MM. HG		CARDIAC OUTPUT, CC. PER MINUTE	
			Before thiamine	Change at minimum arterial pres- sure	Before thiamine	Change at minimum arterial pres- sure
50	33	pH 7.26*	120.3	No change	415.5	No change
100	42	7.25	122.4	No change	347.8	No change
125	—	—	114.6	No change	298.6	No change
250	83	7.18	117.5	-0.5	420.9	-20.9
500	147	7.14	131.0	-3.0	313.7	-20.8
750	441	6.62	125.0	-10.0	201.5	-14.2
1000	464	6.56	148.1	-21.0	233.4	-15.6
2000	1948†	5.40	132.3	-49.0	310.0	-123.3

* The hydrogen ion concentration of whole blood was pH 7.32.

† This high value was the result of several previous additions of thiamine.

heart-lung preparation, it is evident that thiamine does not exert an effect on the heart except in doses far greater than those which, in the intact dog, cause a fall in blood pressure. It is likely that this effect may be attributed to the acidity of the blood.

As has been shown (Table 1) thiamine hydrochloride in doses sufficient to produce respiratory paralysis and decrease in blood pressure, does not alter the pH of blood. It seems evident that in the absence of changes in pH of blood, thiamine does not affect the heart directly to any marked degree. Any effect on the heart contributing to the observed fall in blood pressure must, therefore, originate at some other point, possibly the central nervous system.

DISCUSSION

The most obvious effect of toxic doses of thiamine is respiratory depression or paralysis. There seems, at the moment, to be no valid reason to question the assumption that such an effect is central in origin.

In addition, large doses of thiamine cause a pronounced fall in blood pressure, which is transitory if artificial respiration is maintained.

Any fall may be due to several factors, such as a decrease in blood viscosity and blood volume, or to a decrease in peripheral resistance brought about by vasodilation, or a decrease in the rate or efficiency of the heart beat. We did not study blood viscosity and blood volume; that they might contribute to the observed changes in blood pressure has not been ruled out, but this seems unlikely, since they could not change to a marked extent in the short time required to obtain the full effect of thiamine.

The fall in blood pressure must, therefore, be due to vasodilation and/or a decrease in cardiac efficiency.

We have seen that thiamine has no particular vasodilating effect in the rabbit ear, unless its solutions are acidic. Therefore, thiamine, *per se*, does not act on the blood vessels. However, in the intact dog, vasodilation occurs with either acidic or neutralized thiamine solutions in the absence of significant changes in the pH of the blood. The action of thiamine in the intact animal, is, therefore, not a pH effect, but an effect of thiamine itself. Since thiamine does not appear to act on the blood vessels directly, there remains the alternative that it might depress the vasoconstrictor centers.

In the turtle heart and the dog heart-lung preparation, most of the changes produced by thiamine could be traced to the effect of the acidity of the thiamine solutions. In the intact dog, where no shift in pH is produced by thiamine, there is, nevertheless, a depression of the heart. These results can only indicate that thiamine does not act directly on the heart, but as in the case of the blood vessels, exerts its effect elsewhere.

The meaning of the deep inverted T-wave of the electrocardiogram is not clear to us. It could indicate a direct effect of thiamine on the heart, or an indirect effect due to depressed coronary flow as a result, perhaps, of decreased blood pressure, or it might indicate an effect on the heart originating outside of the heart itself.

We are aware of the dangers of applying results obtained from one species directly to other species. It seems to us that the consistency of results obtained from the dog, rabbit and turtle make general conclusions quite valid.

SUMMARY AND CONCLUSIONS

1. Thiamine hydrochloride solutions, in single intravenous injections to dogs, causes respiratory arrest, marked but transitory fall in blood pressure, bradycardia, transitory vasodilation, and transitory changes in the electrocardiogram.

2. Respiratory arrest is the immediate cause of death. When artificial respiration is maintained until the concentration of thiamine in the blood falls to tolerable levels, spontaneous respiration is resumed, and the dog recovers. Respiratory arrest is presumed to be central in origin.

3. The fall in blood pressure is due to vasodilation and slowing of the heart rate.

4. In the isolated perfused rabbit ear, vasodilation is due to the acidity, and thiamine, *per se*, has little or no direct effect on the blood vessels. In the intact animal, thiamine solutions do not cause a significant change in the pH of blood, nevertheless vasodilation occurs. This indicates that vasodilation is probably not due to direct action on blood vessels, but may be central in origin.

5. A depression of the isolated turtle heart perfused with Ringer plus thiamine is due, essentially, to the acidity of thiamine hydrochloride solutions. In systems where the buffering system of the blood prevents changes of pH and where the central nervous system is not functioning (dog heart-lung preparation) thiamine hydrochloride solutions are without marked effect. In contrast, in systems with the central nervous system intact, moderately strong thiamine hydrochloride solutions cause no change in pH but do cause a slowing of the heart, which probably contributes to the fall in blood pressure. Thus, thiamine itself has no direct effect on the heart, but may affect the heart indirectly, possibly through the central nervous system.

REFERENCES

1. FOA, P. P., SMITH, J. A., AND WEINSTEIN, H. R.: Arch. Biochem., 13: 449, 1947.
2. MILLS, C. A.: J. A. M. A., 116: 2101, 1941.
3. LAWS, C. L.: J. A. M. A., 117: 176, 1941.
4. SCHIFF, L.: J. A. M. A., 117: 609, 1941.
5. EISENSTADT, W. S.: Minnesota Med., 25: 861, 1942.
6. MOLITOR, H.: Federation Proc., 1: 309, 1942.
7. LEITNER, Z. A.: Lancet, 2: 474, 1943.
8. STEIN, W., AND MORGENSTEIN, M.: Ann. Int. Med., 20: 826, 1944.
9. REINGOLD, I. M., AND WEBB, F. R.: J. A. M. A., 130: 491, 1946.
10. HECHT, G., AND WEESE, H.: Klin. Wchnschr., 16: 414, 1936.
11. MOLITOR, H., AND SAMPSON, W. L.: E. Merck's Jahresbericht, 1936.
12. STERN, E. L.: Am. J. Surg., 39: 495, 1938.
13. HALEY, T. J., AND FLESHER, A. M.: Science, 104: 567, 1946.
14. DIAS, M. V.: Science, 105: 211, 1947.
15. HENNESSEY, D. J., AND CERECEDO, L. R.: J. Am. Chem. Soc., 61: 179, 1939.
16. SMITH, J. A.: Science, 106: 622, 1947.

THE ADDICTION LIABILITY OF SOME DRUGS OF THE METHADON SERIES

HARRIS ISBELL AND ANNA J. EISENMAN

The Research Division, U. S. Public Health Service Hospital, Lexington, Kentucky

Received for publication March 15, 1948

Since racemic methadon has been shown to be an addicting drug (1, 2, 3), a study of the addiction liability of some other members of the methadon series was undertaken. The following compounds have been studied: The optical isomers of methadon¹ (dextro- and levorotary 6-dimethylamino-4-4-diphenyl-3-heptanone), racemic methadol² (6-dimethylamino-4-4-diphenyl-3-heptanol, the alcoholic analogue of methadon); and racemic isomethadon³ (6-dimethylamino-5-methyl-4-4-diphenyl-3-hexanone).

Pharmacological data on these compounds are still fragmentary. Dextro- and levomethadon are approximately equal in toxicity when administered subcutaneously to mice (4), but the analgesic potency of levomethadon in mice is twice that of racemic methadon, while the analgesic potency of the d-isomer is less than $\frac{1}{10}$ the potency of the racemate. Methadol is only $\frac{1}{3}$ as toxic as racemic methadon for mice, but is only $\frac{1}{10}$ as active in producing analgesia. It does not produce a Straub reaction (4), and seems to be chiefly a depressant drug in mice. The isomethadon used in these experiments was supplied by one laboratory³ and was stated to be racemic 6-dimethylamino-5-methyl-4-4-diphenyl-3-hexanone hydrochloride monohydrate. This material irregularly produces a Straub reaction in mice (4), and is $\frac{2}{3}$ as toxic as methadon (4). Two mgm./kgm. of isomethadon has no effects on the hindlimb reflexes of chronic spinal dogs (5). Five mgm./kgm. produces effects identical with those of morphine or methadon—depression of the flexor and crossed extensor reflexes, exaggeration of the extensor thrust reflex, and variable effects on the knee jerk (5). Two mgm./kgm. elevates the tooth pain-reaction threshold of dogs 50 to 100 per cent (5).

In all our experiments, the hydrochlorides of the various drugs were dissolved in physiological saline and all doses were injected subcutaneously, except as noted below.

EFFECTS OF SINGLE DOSES OF THE DRUGS ON FORMER MORPHINE ADDICTS. Twelve former morphine addicts, who volunteered for the experiments, agreed to take single doses of the various drugs. These men rested quietly in bed for one hour after which pulse and respiratory rates, rectal temperatures, and blood pressures were determined 3 times at intervals of 30 minutes for 4 to 6 hours. The men were required to lie quietly in bed throughout the experiment, but were not permitted to fall asleep. They were observed carefully for evidences of seda-

¹ Supplied through the courtesy of Merck and Company, Rahway, New Jersey.

² Supplied by Drs. N. B. Eddy and Lyndon F. Small, National Institute of Health, Bethesda, Maryland.

³ Supplied by the Mallinckrodt Chemical Company, St. Louis, Missouri.

4. In the isolated perfused rabbit ear, vasodilation is due to the acidity, and thiamine, *per se*, has little or no direct effect on the blood vessels. In the intact animal, thiamine solutions do not cause a significant change in the pH of blood, nevertheless vasodilation occurs. This indicates that vasodilation is probably not due to direct action on blood vessels, but may be central in origin.

5. A depression of the isolated turtle heart perfused with Ringer plus thiamine is due, essentially, to the acidity of thiamine hydrochloride solutions. In systems where the buffering system of the blood prevents changes of pH and where the central nervous system is not functioning (dog heart-lung preparation) thiamine hydrochloride solutions are without marked effect. In contrast, in systems with the central nervous system intact, moderately strong thiamine hydrochloride solutions cause no change in pH but do cause a slowing of the heart, which probably contributes to the fall in blood pressure. Thus, thiamine itself has no direct effect on the heart, but may affect the heart indirectly, possibly through the central nervous system.

REFERENCES

1. FOA, P. P., SMITH, J. A., AND WEINSTEIN, H. R.: Arch. Biochem., **13**: 449, 1947.
2. MILLS, C. A.: J. A. M. A., **116**: 2101, 1941.
3. LAWS, C. L.: J. A. M. A., **117**: 176, 1941.
4. SCHIFF, L.: J. A. M. A., **117**: 609, 1941.
5. EISENSTADT, W. S.: Minnesota Med., **25**: 861, 1942.
6. MOLITOR, H.: Federation Proc., **1**: 309, 1942.
7. LEITNER, Z. A.: Lancet, **2**: 474, 1943.
8. STEIN, W., AND MORGENSTEIN, M.: Ann. Int. Med., **20**: 826, 1944.
9. REINGOLD, I. M., AND WEBB, F. R.: J. A. M. A., **130**: 491, 1946.
10. HECHT, G., AND WEESE, H.: Klin. Wchnschr, **16**: 414, 1936.
11. MOLITOR, H., AND SAMPSON, W. L.: E. Merck's Jahresbericht, 1936.
12. STERN, E. L.: Am. J. Surg., **39**: 495, 1938.
13. HALEY, T. J., AND FLESHER, A. M.: Science, **104**: 567, 1946.
14. DIAS, M. V.: Science, **105**: 211, 1947.
15. HENNESSEY, D. J., AND CERECEDO, L. R.: J. Am. Chem. Soc., **61**: 179, 1939.
16. SMITH, J. A.: Science, **106**: 622, 1947.

period of observation. In instances in which only slight or no effects were seen, another and larger dose of the same drug was given or, in some instances, a different drug was administered. The first pair of men who were withdrawn from morphine received a small dose of the drug being tested and, if there was no relief or only partial relief of the abstinence symptoms, the dose was increased on sub-

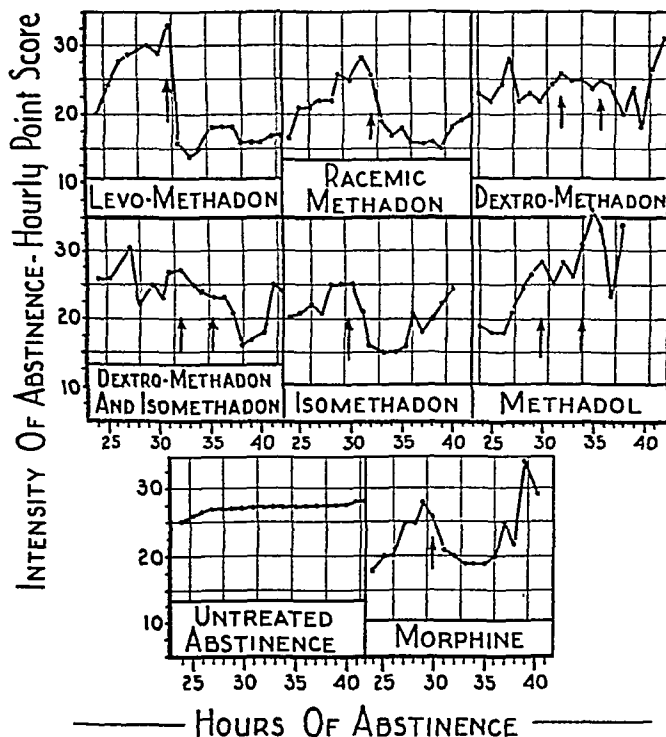


FIG. 1. EFFECTS OF DRUGS OF METHADON SERIES ON ABSTINENCE FROM MORPHINE

Ordinates: average intensity of abstinence expressed in hourly points (6). Abcissae: hours of abstinence. Arrows indicate injection of drugs. 10 subjects received levomethadon, racemic methadon, and isomethadon; 4 received 2 doses of dextromethadon; 6 received one dose of dextromethadon followed by one dose of isomethadon; 4 received one dose of morphine. Course of untreated abstinence taken from data of Himmelsbach (6).

sequent trials until an effective level had been found, or until it was certain the drug would be without effect.

Doses of 6.25 mgm. to 22.5 mgm. of levomethadon (average 11 mgm.) caused a striking diminution in the intensity of the abstinence (fig. 1) which persisted throughout the period of observation. The degree of relief of abstinence by levomethadon was greater and persisted longer than the relief afforded by 30 mgm. of morphine administered to 4 of the same subjects during subsequent withdrawals. Levomethadon was approximately twice as potent as racemic

tion and euphoria by especially trained attendants. The experiments were conducted on 2 men at a time. The first pair received a small dose of the drug being tested. If no untoward effects were observed, the dose was raised in subsequent tests until there was evidence of marked effects, or until such a large dose had been given without effect that there seemed to be no reason to increase the dosage further. Two weeks after the initial tests the men were recalled and given various doses of the drugs intravenously after which they were carefully observed for sedation and euphoria.

Dextromethadon in doses of 15 to 90 mgm., methadol in doses of 30 to 100 mgm., and isomethadon in doses from 15 to 45 mgm. subcutaneously, had no consistent effects on blood pressures, respiratory rates, or pulse rates in these post-addicts. No evidence of sedation or euphoria was observed after any of the 3 drugs. The reaction of the men was generally one of disappointment, and they frequently said that they had not received any drug. The men who received the largest doses of isomethadon complained of being nervous and said the drug had effects similar to those of benzedrine. When these drugs were injected intravenously, no evidence of sedation or euphoria was noted after dextromethadon or methadol, but 30 mgm. or more of isomethadon intravenously regularly produced euphoria which was manifested by increased talkativeness, etc. The men who received isomethadon seemed to be slightly exhilarated rather than sedated. Five to 15 mgm. of levomethadon was regularly followed by a depression of respiratory rate of 4 to 6 per minute, depression of the pulse rate of 5 to 10 per minute, and by a slight depression of systolic blood pressure. Definite evidence of both sedation and euphoria, which was quite marked with 15-mgm. doses, was noted after levomethadon. The euphoria after levomethadon was not evident for 1 to 1½ hours after the injection and persisted for as long as 36 to 48 hours. The post-addicts could not distinguish the subjective effects of levomethadon given intravenously from those of heroin or dilaudid. Miosis was never observed after methadol or dextromethadon, but was regularly seen after levomethadon and isomethadon. Nausea and vomiting never occurred after dextromethadon, methadol, or isomethadon. Nausea followed levomethadon (15-mgm. dose) in 3 instances and vomiting occurred once.

EFFECTS OF SINGLE DOSES OF THE DRUGS ON THE MORPHINE ABSTINENCE SYNDROME. The effect of single doses of the various drugs on the morphine abstinence syndrome was studied by administering the drugs subcutaneously to 10 men strongly addicted to morphine (stabilization doses of 240 to 480 mgm. of morphine daily) 28 to 32 hours after they had received their last dose of morphine, and while they were showing signs of moderate to severe abstinence. Observations of the intensity of abstinence were made hourly from the 24th to the 28th to the 32d hour after withdrawal and the results scored by the hourly point system of Himmelsbach (6). Observations were continued for 10 hours after the test dose was given. The subjects were then returned to their usual dose of morphine for 10 days after which they were again used in testing another drug. In instances in which definite alleviation of the abstinence syndrome followed the test dose of the drug, no more medication was given throughout the

period of observation. In instances in which only slight or no effects were seen, another and larger dose of the same drug was given or, in some instances, a different drug was administered. The first pair of men who were withdrawn from morphine received a small dose of the drug being tested and, if there was no relief or only partial relief of the abstinence symptoms, the dose was increased on sub-

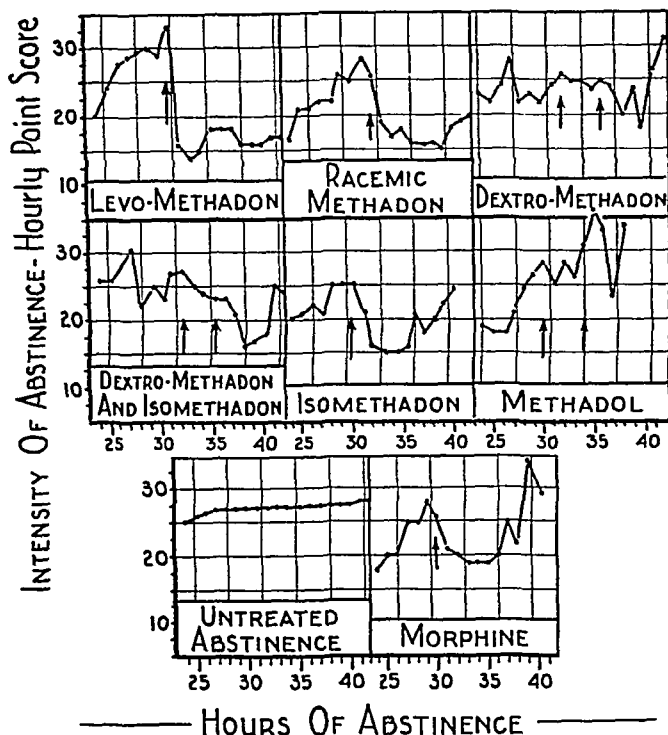


FIG. 1. EFFECTS OF DRUGS OF METHADON SERIES ON ABSTINENCE FROM MORPHINE

Ordinates average intensity of abstinence expressed in hourly points (6). Abscissae: hours of abstinence. Arrows indicate injection of drugs. 10 subjects received levomethadon, racemic methadon, and isomethadon, 4 received 2 doses of dextromethadon; 6 received one dose of dextromethadon followed by one dose of isomethadon, 4 received one dose of morphine. Course of untreated abstinence taken from data of Himmelsbach (6).

sequent trials until an effective level had been found, or until it was certain the drug would be without effect.

Doses of 6.25 mgm. to 22.5 mgm. of levomethadon (average 11 mgm.) caused a striking diminution in the intensity of the abstinence (fig 1) which persisted throughout the period of observation. The degree of relief of abstinence by levomethadon was greater and persisted longer than the relief afforded by 30 mgm. of morphine administered to 4 of the same subjects during subsequent withdrawals. Levomethadon was approximately twice as potent as racemic

methadon in reducing the intensity of abstinence from morphine. About 1 mgm. of levomethadon for each 9 mgm. of the stabilization dose of morphine was required to reduce the intensity of abstinence from approximately 25 to 15 Himmelsbach hourly points. About 1 mgm. of racemic methadon for each 4 mgm. of the stabilization dose of morphine were required to effect a comparable reduction in abstinence. Dextromethadon in doses of 30 to 90 mgm. had no detectable effect on the intensity of abstinence. Apparently, all the effect of racemic methadon in relieving abstinence from morphine is due to the levorotary isomer. Sixty to 120 mgm. of methadol did not influence the course of abstinence in any way.

Sixty to 90 mgm. of isomethadon (average 76.5 mgm.) reduced the intensity of abstinence about as much as did 30 mgm. of morphine. The duration of relief was 4 to 6 hours. About 1 mgm. of isomethadon for each 1.33 mgm. of the stabilization dose of morphine was required to reduce the intensity of abstinence from 25 to 15 points.

SUBSTITUTION OF LEVOMETHADON FOR MORPHINE. One mgm. of levomethadon was substituted for each 8 mgm. of the stabilization dose of morphine in 7 men who were strongly addicted to morphine (60 mgm. of levomethadon for 480 mgm. of morphine sulfate daily). No signs of abstinence appeared following the substitution (fig. 2) and the Himmelsbach daily point score (7) did not rise. After the substitution had been effected, the dose of levomethadon was progressively reduced in an attempt to determine a stabilization dose. No signs of abstinence appeared so that a stabilization dose could not be determined. When the total daily dosage of levomethadon had been reduced to 20 mgm. daily (1 mgm. of levomethadon for each 24 mgm. of the stabilization dose of morphine) the dosage was not lowered any further.

After 10 days substitution, the administration of levomethadon was abruptly and completely discontinued. Observations for signs of abstinence were calculated according to the daily point score system of Himmelsbach (7). The patients had no complaints for the first 2 to 3 days of abstinence; thereafter they complained of weakness, peculiar sensations in the abdomen, anxiety, anorexia and insomnia. The course and type of abstinence in 4 of the men was similar to those previously described after substitution of racemic methadon for morphine (1). These 4 patients showed practically no signs of disturbances of autonomic function, did not vomit, or have diarrhea. More signs of disturbed autonomic function were observed in the remaining 3 patients than we had seen in our experience with racemic methadon, but the incidence of these signs was not nearly so great as after the withdrawal of morphine for 38 to 42 hours in the same patients, and the signs were not consistently present from hour to hour in the same men. The average intensity of abstinence in all 7 men, as judged by the Himmelsbach daily point score (fig. 2), was similar to that seen after the withdrawal of racemic methadon (1). The point score rose slowly and did not exceed 20 points at the end of 14 days of observation. Average rectal temperatures, pulse rates, respiratory rates, and morning systolic blood pressures were elevated above addiction means from the 3d to the 14th day of abstinence.

SUBSTITUTION OF ISOMETHADON FOR MORPHINE. One mgm. of isomethadon

was substituted for each 1.33 mgm. of the stabilization dose of morphine (360 mgm. of isomethadon for 480 mgm. of morphine) in 5 cases strongly addicted to morphine. The substitution ratio was the largest possible with the amount of isomethadon available at the time of the experiment, and was chosen because of the high doses of isomethadon required to produce good relief of abstinence from

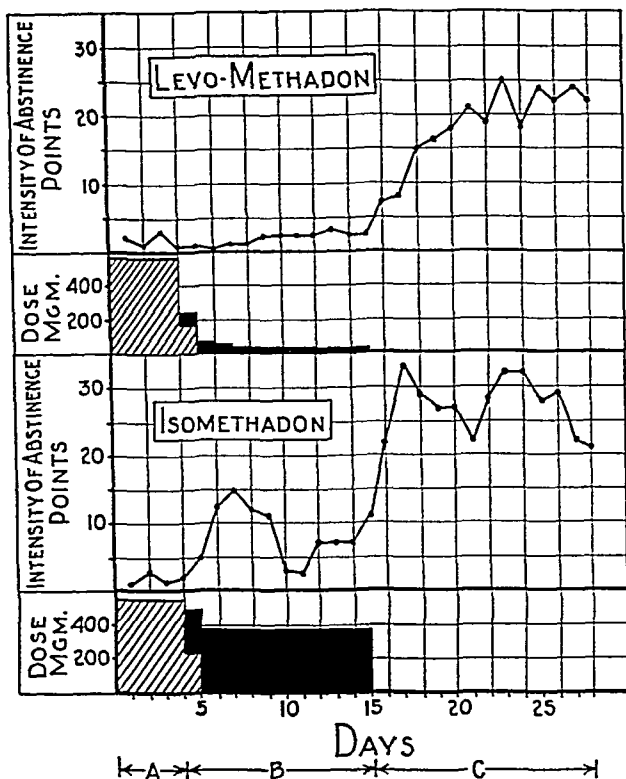


FIG. 2. SUBSTITUTION OF LEVOMETHADON AND ISOMETHADON FOR MORPHINE

Intensity of abstinence is expressed in daily points (7). A. Period of stabilization on morphine. B. Period of substitution of isomethadon or levo-methadon for morphine. C. Period of abstinence.

morphine. Following the substitution of isomethadon mild signs of abstinence appeared in all 5 men. Three of the patients showed lacrimation and rhinorrhea; 2 vomited. Temperatures, pulse rates, and respiratory rates were elevated above preaddiction values in all 5 subjects. The Himmelsbach daily point scores were elevated to 15 points on the 3d day of substitution and remained elevated throughout the substitution period (fig. 2). While this substitution was not entirely adequate, it is possible that if larger amounts of isomethadon had been available all signs of abstinence might have been suppressed.

Twelve to 18 hours after abrupt withdrawal of isomethadon, all 5 men began to exhibit yawning, lacrimation, rhinorrhea, mydriasis, and gooseflesh. These signs are identical with those seen after the withdrawal of morphine. The incidence of disturbances in autonomic function increased until the 24th to the 36th hour of abstinence. Restlessness, vomiting, diarrhea, fever, hyperpnea, tachycardia, and elevation of systolic blood pressure were observed. The men's complaints were typical of the morphine abstinence syndrome. The intensity of abstinence, as judged by the Himmelsbach daily point score, was less severe than abstinence from morphine but distinctly more severe than abstinence from racemic or levomethadon. The average point score reached a maximum of 33 points on the 2d day of abstinence, declined slowly and irregularly thereafter, but was still elevated above 20 points at the end of 14 days of observation.

DIRECT ADDICTION TO ISOMETHADON. Ten post-addicts, who had been abstinent from morphine for 3 months or more, volunteered to take isomethadon experimentally for 42 to 59 days. They had no significant physical defects and were all diagnosed psychiatrically as having psychopathic personalities. Isomethadon was administered subcutaneously 4 times daily. The initial dosage was 80 mgm. daily and was increased as rapidly as tolerance permitted to 270 to 360 mgm. daily. The precautions listed by Kolb and Himmelsbach (7) were followed and observations for signs of abstinence (7) were made 3 times daily, by observers especially trained in Himmelsbach's methods, for 4 days before addiction was begun, throughout addiction, and for 14 days after withdrawal. Blood counts, urinalyses, cephalin-cholesterol flocculation tests, and fasting blood sugars were obtained before addiction, once every 2 weeks during addiction, and on the 2d, 7th and 10th days of withdrawal.

When the men first began to receive the drug, they became somewhat exhilarated after the 2d to the 4th dose, laughed and joked with each other, and were much more active than before beginning addiction. Total hours of sleep per day were reduced in the first few days of addiction. Some men did not sleep at all in the first 48 hours of addiction. After a day or two most of the subjects complained of nervousness, which, in two instances, became so severe as to require reduction in dosage or omission of doses. After about a week most of the men gradually became less exhilarated, and, as the dosage was increased, they began to exhibit signs of sedation, stayed in bed more, and slept more than before beginning the experiment. They never showed as marked a degree of sedation as did men addicted to methadon (1) or morphine. Seven of the patients continued to work throughout the experiment and there was little deterioration in their performance. They did not neglect their personal appearance and quarters as much as did men addicted to methadon or morphine. After the initial phase of exhilaration had passed, the men were disappointed with the effects of the drug and said that it was about equal to codeine in euphoric effect. In the 2d and 3d weeks of addiction they frequently requested elevations in the dosage, but thereafter asked for no more increases and seemed to tolerate further elevation in dosage as a necessary evil. None of the men asked to be dropped from the experiment, and all maintained their good humor with each other and with the attendants throughout the addiction period.

Early in the addiction period, changes in pulse and respiratory rates, and resting systolic blood pressure were less marked than during addiction to either morphine or methadon. As the dosage was elevated, systolic blood pressure fell 7 to 10 mm. Hg, the pulse rate was slowed 15 per minute and the respiratory rate 6 per minute. Rectal temperatures were unchanged. Caloric intake and body weight declined. All the patients complained of severe constipation. Miosis was noted in all men in the first week of the experiment and persisted throughout addiction in 3 subjects. No serious toxic effects were observed other than the development of induration, inflammation, and numbness of the skin over the injection sites. Cephalin-cholesterol flocculation tests, urinalyses, fasting blood sugars and total white blood cell counts were unchanged throughout addiction. Occasionally eosinophilia of 5 to 11 per cent was noted in differential white blood cell counts. A mild normochromic, normocytic anemia developed in the second month of addiction. Red blood cell counts fell from preaddiction levels of 4.36–4.97 millions in the preaddiction period to 3.59–4.38 millions per cu. mm. in the second month of addiction. Hemoglobin values declined from 13.8–15.2 grams to 10.8–12.6 grams per 100 cc. The anemia was improving at the end of the addiction period.

Isomethadon was abruptly withdrawn from one man after 42 days addiction and from the remainder after 56 days addiction. An abstinence syndrome ensued which was indistinguishable from the abstinence syndrome described under Substitution of Isomethadon, except that the onset of abstinence was more variable in the group of men who had been directly addicted to the drug than in the substitution group. Four men exhibited well developed signs of abstinence 12 hours after the last dose; 5 did not show signs until the 18th hour of abstinence. Qualitatively, the isomethadon abstinence syndrome resembled withdrawal of morphine more than withdrawal of methadon. Quantitatively, it was not as severe as abstinence from morphine, became manifest and subsided at about the same rate (fig. 3). Abstinence from isomethadon began sooner, was more severe, and declined more rapidly than abstinence from methadon.

DISCUSSION. Dextromethadon and methadol do not appear to possess addiction liability and, since they are not effective analgesics, they need not be considered further. Levomethadon definitely has addiction liability. It produces intense euphoria in former morphine addicts, relieves abstinence from morphine, and suppresses signs of abstinence when substituted for morphine in cases addicted to that drug. Following withdrawal of levomethadon after substitution for morphine, a definite abstinence syndrome appears which is quite similar to the abstinence syndrome after withdrawal of racemic methadon. Levomethadon appears to account for all of the addiction liability of racemic methadon as well as for all of its analgesic action.

Isomethadon must also be classed as an addictive substance. It is much less active in inducing euphoria than are methadon and morphine and also does not seem to produce as marked a degree of habituation (emotional dependence) as do methadon and morphine. It will, however, relieve abstinence from morphine and suppresses, in part at least, signs of abstinence when substituted for morphine. Definite evidence of physical dependence of a moderate grade was seen

after withdrawal of isomethadon both after substitution for morphine and after direct addiction for 42 to 59 days.

The quick onset and morphine-like course of the isomethadon abstinence syndrome is strikingly different from the slow onset and mild, slow course of absti-

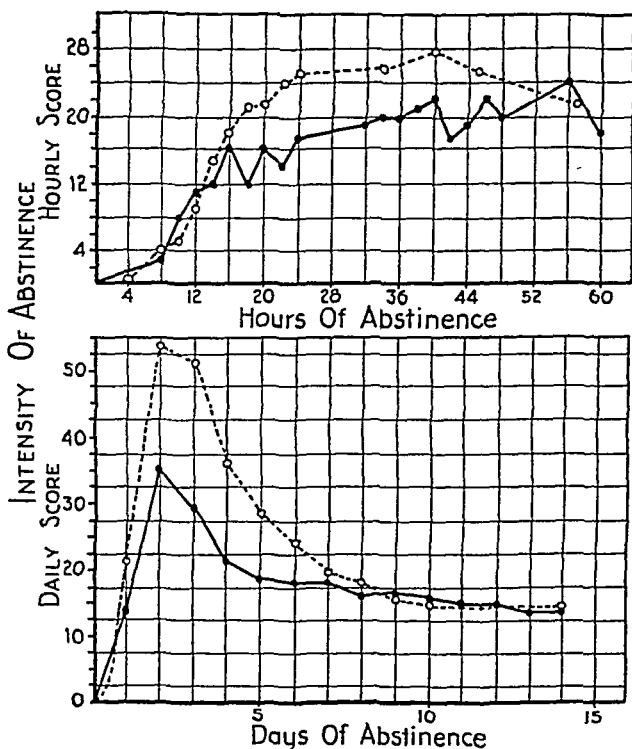


FIG. 3. INTENSITY OF ABSTINENCE FOLLOWING WITHDRAWAL OF ISOMETHADON AFTER EXPERIMENTAL ADDICTION FOR 42 TO 59 DAYS

Averages of 10 cases. Upper curve: Onset of abstinence from isomethadon as compared to onset from morphine. Intensity expressed in hourly points (6). Lower curve: Course of abstinence from isomethadon compared to abstinence from morphine. Intensity of abstinence expressed in daily points (7). Solid line shows intensity of abstinence from isomethadon. Dotted line shows intensity of abstinence from morphine based on 65 cases of Kolb and Himmelsbach.

nence from methadon. There is as yet no adequate explanation for the diversity of these two abstinence syndromes, but the differences might possibly be accounted for by differences in the storage or rate of metabolism of the two drugs.

SUMMARY

1. Methadol and dextromethadon do not produce euphoria in post-addicts and will not relieve abstinence from morphine.

2. Levomethadon produces euphoria in post-addicts, relieves abstinence from morphine, and suppresses signs of physical dependence when substituted for morphine in cases strongly addicted to morphine. Following withdrawal of levomethadon, after substitution for morphine, an abstinence syndrome develops which is identical with the syndrome of abstinence from racemic methadon. Levomethadon accounts for all the addiction liability of racemic methadon.

3. Isomethadon in sufficient dose intravenously produces euphoria in post-addicts, relieves abstinence from morphine, and partially suppresses signs of abstinence when substituted for morphine in cases addicted to that drug. Following withdrawal of isomethadon, after substitution for morphine, or after direct addiction of former morphine addicts to isomethadon for 42 to 59 days, an abstinence syndrome develops very rapidly. The isomethadon abstinence syndrome resembles abstinence from morphine more than abstinence from methadon. Isomethadon is an addicting drug.

BIBLIOGRAPHY

- 1 ISBELL, H , WIKLER, A , EDDY, N B , WILSON, J L , AND MORAN, C F J A M A , 135: 888, 1947
- 2 ISBELL, H , LISLAMAN, A J , WIKLER, A , DAINGERFIELD, M , AND FRANK, K Fed Proc , 6: 264, 1947 (abstract)
- 3 WIKLER, A , AND FRANK, K Fed Proc , 6: 384, 1947 (abstract)
- 4 EDDY, N B Personal communication
- 5 WIKLER, A Personal communication
6. HIMMELSBACH, C K THIS JOURNAL , 67: 239, 1939
- 7 KOLB, L , AND HIMMELSBACH, C K Am J Psychiat , 94: 759, 1938

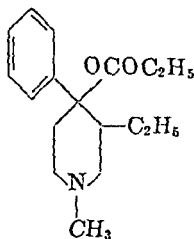
PHARMACOLOGICAL STUDIES ON ANALGESIC PIPERIDINE DERIVATIVES

LOWELL O. RANDALL AND G. LEHMANN

Pharmacology Lab., Hoffmann-La Roche, Inc., Nutley, New Jersey

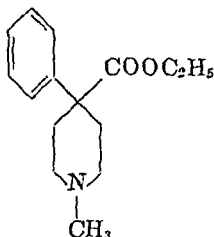
Received for publication March 15, 1948

The comprehensive program of synthesizing new analgesics initiated and carried out by Lee and coworkers (1-3) has yielded compounds with considerable potency (4). Chemically they are 1-, 4-, 4-substituted piperidines. It is well known that isomerism has great influence on pharmacological potency. Pursuing their program Lee and associates introduced an additional substituent into the 3-position which of necessity yielded different isomers. We have studied the pharmacological properties of a number of such 3-substituted piperidine derivatives in addition to a series in which substitution on the 3-carbon atom was omitted. The structural relationship of the former to meperidine and methadon is demonstrated below:



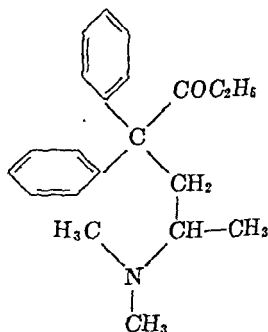
Nu-1932

1-methyl-3-ethyl-4-phenyl-4-propionyloxy piperidine.



Meperidine

1-methyl-4-phenyl-4-carboxylic acid ethyl ester.



Methadon

6-dimethylamino-4,4-diphenyl-3-heptanone.

METHODS. Toxicity determinations of the 3-substituted piperidine derivatives were made on albino mice, rats and rabbits by various routes. The LD₅₀ values and their standard errors were calculated by a graphical method (5). Toxicity of the remaining compounds was determined in mice by subcutaneous administration.

Analgesia was measured by a modification of the method of Hardy, Wolff and Goodell (6) which was previously described (4). The normal reaction time to a constant heat stimulus was measured in triplicate. After subcutaneous or oral administration of the compound to be tested, the reaction time was measured at 30 minute intervals for two hours. The maximal increase in per cent was used as a measure for analgesic potency. Preliminary tests were carried out on five rats for each dose level and larger numbers of animals were used for determining analgesic potency of the more active compounds. The method of Bliss and Marks (7) was applied for calculating the potency in terms of morphine sulphate or methadon.

Spasmolytic action was estimated on isolated rabbit's and guinea pig's intestine suspended in Locke's solution against spasm induced by acetylcholine, histamine and barium and compared with the effects of atropine, epinephrine and papaverine respectively. In addition, the action of the more potent compounds after intravenous administration has been studied on spontaneous jejunal motility in two trained unanesthetized dogs with Thiry-Vella fistulas. Eighteen injections were made in 9 experiments.

The effect on respiration was observed in rabbits sedated with 15 mg./kg. of pentobarbital intravenously. Using a two-valve system, the expired air was collected in a calibrated spirometer provided with a damping vane in oil (8) to obtain adequate sensitivity. Respiratory rate and minute volume were observed before and at constant intervals after the intravenous injection of the more potent preparations. At least three animals were used for each dose level.

RESULTS. *Analgesic action and toxicity.* The data on analgesic activity and toxicity of the 3-substituted piperidine derivatives in comparison with morphine and methadon are compiled in tables 1 and 2. Of these compounds, Nu-1196 and Nu-1779 are diastereomers and Nu-1831 and Nu-1832 are the resolved optical antipodes of Nu-1779. There is little difference in toxicity among these isomers regardless of species and route of administration but a marked difference in analgesic activity is evident. On subcutaneous administration, the potency of Nu-1196 is about the same as that of morphine and methadon, whereas its diastereomer, Nu-1779, is five times as active. Its levo isomer, Nu-1832, is twice as potent as the dextro isomer, Nu-1831. The 3-ethyl derivative, Nu-1932, is slightly less toxic but has about the same analgesic activity as the 3-methyl isomer, Nu-1779. These compounds are also active when administered orally. The potencies relative to methadon under these experimental conditions are about the same as those obtained after subcutaneous administration.

Figure 1 illustrates the linear relationship which exists between analgesic response and log of dose. If the percent increase in reaction time obtained after subcutaneous administration (table 1) is plotted against the log of the dose, the following order of decreasing potency is apparent: Nu-1832, Nu-1932, Nu-1779, Nu-1831, morphine sulfate and Nu-1196. The straight lines drawn in figure 1 fit the experimentally found points fairly well, considering that the error of any individual assay is about 20 percent and that a four-fold increase of the minimum analgesic dose is about the limit of the response range.

The results obtained from a tolerance test with three of the more potent analgesic agents in comparison with morphine are plotted graphically in figure 2. The compounds were injected daily by the subcutaneous route into groups of 10 rats each and analgesic tests were carried out after the first injection and at weekly intervals thereafter for a period of seven weeks. The doses used were as follows: morphine, 6 mg./kg.; Nu-1196, 6 mg./kg.; Nu-1832, 1 mg./kg.; and Nu-1932, 1 mg./kg. From the curves it is evident that tolerance develops rapidly to morphine and Nu-1932, less rapidly to Nu-1832, and not at all to Nu-1196 within a period of seven weeks.

Growth rates which were observed during the tolerance test showed that the average weights of the treated rats including those which had received morphine were about 15 percent lower than the controls at the end of the seven weeks

TABLE 1

Analgesic effects of 1,3-dialkyl-4,4-disubstituted piperidine compounds

NU-	NAME	ANALGESIA SUBCUTANEOUS				ANALGESIA ORAL			
		Dose	No. rats	Per cent increase	Potency: per cent of morphine \pm s.e.	Dose	No. rats	Per cent increase	Potency: per cent of Methadon \pm s.e.
		mg./kg.				mg./kg.			
1196	dl - α - 1,3 - Dimethyl - 4 - phenyl - 4 - propionyxy piperidine hydrochloride	1.5 3.0 6.0	15 15 15	28 61 156	97 \pm 16	5 10 20	5 10 10	15 66 122	140 \pm 45
1779	dl - β - 1,3 - Dimethyl - 4 - phenyl - 4 - propionyxy piperidine hydrochloride	.25 .50 1.0	35 35 35	21 75 130	550 \pm 51	2 4 8	10 10 10	49 105 108	420 \pm 80
1831*	d - β - 1,3 - Dimethyl - 4 - phenyl - 4 - propionyxy piperidine d-acid tartrate (higher melting enantiomorph)	.5 1.0 2.0	15 15 15	33 89 171	350 \pm 22	2 4 8	10 10 10	56 77 128	390 \pm 81
1832*	1 - β - 1,3 - Dimethyl - 4 - phenyl - 4 - propionyxy piperidine d-acid tartrate (lower melting enantiomorph)	.25 .50 1.0	15 15 15	38 116 170	790 \pm 51	2 4 8	10 10 10	59 140 154	505 \pm 93
1215	1,3 - Dimethyl - 4 - cyclohexyl - 4 - propionyxy piperidine hydrochloride	3 6 9	5 10 15	21 40 130	30				
1959	1 - Methyl - 3 - ethyl - 4 - phenyl - 4 - acetoxxy piperidine hydrochloride	3 6 12	15 15 15	41 108 150	43 \pm 19				
1932	1 - Methyl - 3 - ethyl - 4 - phenyl - 4 - propionyxy piperidine hydrochloride	.25 .5 1.0	10 10 20	23 100 146	640 \pm 120	2 4 8	10 10 10	75 76 120	430 \pm 88
	Morphine	1.5 3.0 6.0	85 85 85	37 70 146	100				
	Methadon	1.5 3.0 6.0	5 5 5	30 88 144	99 \pm 25	5 10 20	10 10 10	11 26 64	100

period. The weight loss was presumably due to the reduced food intake. In addition, red and white cell counts and hemoglobin determinations, made at the outset of the tolerance test and seven weeks later, did not reveal any significant

TABLE 2

Toxicity, spasmolytic and respiratory effects of 1,3-dialkyl-4,4-disubstituted piperidine compounds

Nu.	TOXICITY, LD ₅₀ MG./KG. \pm S.E.						SPASMOLYTIC ACTION ON ISOLATED INTESTINE AGAINST				DOSE mg./kg.	RESPIRATION IN RABBITS				
	Mice i.v.	Mice i.p.	Mice s.c.	Rats s.c.	Rats oral	Rabbits i.v.	Acetylcholine (atropine = 1)	Histamine (epinephrine = 1)	Ba (papaverine = 1)	Per cent decrease		Duration min.	Dose which decreases 50 per cent	Ratio to morphine		
										Minute volume					Rate	
1196	32 \pm 5	85 \pm 12	115 \pm 31	50 \pm 8	90	22	1/700	1/200	1/7	1.0 2.0	31 65	26 59	1.5	1.2		
1779	37 \pm 5	88 \pm 27	100 \pm 28	43 \pm 9	90	17	1/1000	1/200	1/10	0.125 0.25	25 59	23 74	0.19	9.5		
1831*	53 \pm 11	104 \pm 26	100 \pm 24				1/1000	1/200	1/5	0.175 0.35	28 50	33 46	0.35	5.1		
1832*	39 \pm 7	77 \pm 28	80 \pm 18				1/1000	1/200	1/10	0.175 0.35	31 72	37 73	0.24	7.5		
1215			175 \pm 40				1/500	1/100	1							
1959		158 \pm 17	200 \pm 40				<1/1000									
1932	57 \pm 16	136 \pm 16	150 \pm 30	90 \pm 15	175	18	1/1000	1/400	1/2	0.5 0.75	45 66	44 79	0.55	3.3		
Morphine	230 \pm 25		360 \pm 18							1.0 2.0	31 54	22 43	1.8	1.0		
Methadon	20 \pm 5		48 \pm 19	45 \pm 16	90					0.5 1.0	36 72	33 73	0.62	2.9		

* The doses of Nu-1831 and Nu-1832 are calculated in terms of the hydrochlorides.

difference between treated and control groups. Macroscopic examination at autopsy did not reveal any pathological organ changes.

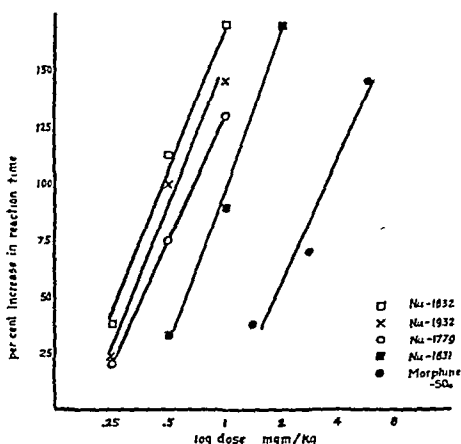


FIG. 1. DOSE-RESPONSE CURVE OF 3-SUBSTITUTED 1-METHYL, 4-PHENYL, 4-PROPIONOXY PIPERIDINES IN RATS
Abscissa: log of dose, mg. per kg.; ordinate: per cent increase in reaction time

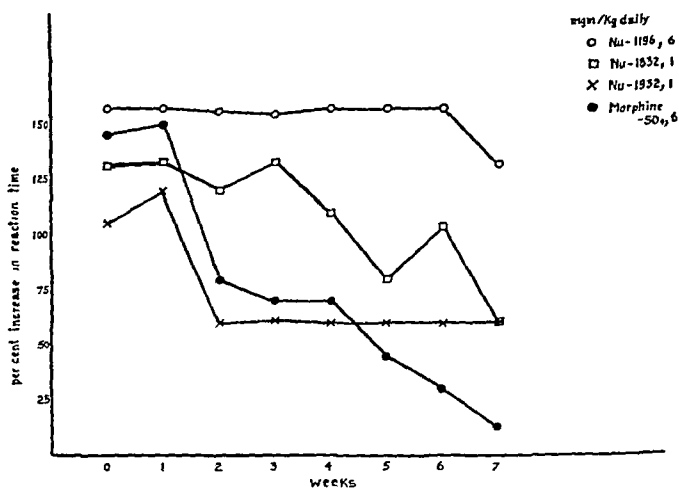


FIG. 2. TOLERANCE TO 3-SUBSTITUTED 1-METHYL, 4-PHENYL, 4-PROPIONOXY PIPERIDINES IN RATS
Abscissa: weeks; ordinate: per cent increase in reaction time

The results obtained on analgesic activity and toxicity of the remaining 43 piperidine derivatives are presented in table 3. Only 8 of these compounds show measurable analgesic activity. Of the active 4-cyclohexyl-4-propionoxy deriva-

tives the 1-methyl derivative (Nu-1333) is about two-thirds, the 1-isopropyl derivative (Nu-1406) about one-third and the 1-butyl derivative (Nu-1109) about one-sixth as active as morphine. Only very weak analgesic effects are observed with 5 other derivatives (Nu-1304, Nu-1077, Nu-1056, Nu-1820 and Nu-1334). There is no correlation of the toxicity of these compounds with their analgesic activity.

Action on respiration and arterial pressure. Only the five most active compounds have been tested. Respiratory rate and minute volume of rabbits were diminished by all five preparations (table 2). The doses which decreased minute volume by 50 per cent were calculated. It may be noted that a certain parallelism exists between the degree of respiratory depression and analgesic activity, that is, the more potent analgesics are the more potent respiratory depressants.








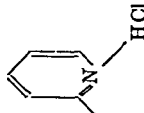

The compounds listed in table 1, when injected intravenously into dial-urethane anesthetized dogs in doses of 1-4 mg. per kg., produce a moderate fall in arterial pressure and slowing of the heart rate. Tolerance to the depressor effect is frequently observed with all compounds on repeated administration. The inhibitory effect on the heart rate seems to be of central origin since it is abolished by bilateral vagotomy.

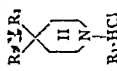
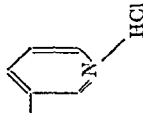
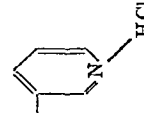
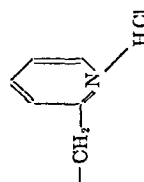
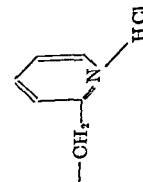
Action on intestinal motility. All compounds if used in sufficiently high concentrations abolish spasm of isolated intestinal strips induced by acetylcholine, histamine or barium chloride. Spasmolytic potency is, however, universally low (tables 2 and 3).

Three of the more potent analgesics, Nu-1196, Nu-1779 and Nu-1932, injected intravenously in a dose of 1 mg. per kg. into dogs with Thiry-Vella fistulas, produced an immediate rise of intestinal tone which in some instances lasted as long as two hours. A typical record is seen in figure 3. The discrepancy between "in vitro" and "in vivo" action of these compounds suggests that the latter is due to central vagal stimulation.

The stimulation of the intestine in the whole animal indicated the possibility of an inhibitory action of these compounds on cholinesterase, since it has been shown (9, 10, 11) that morphine has some anticholinesterase activity. We have studied the effect on cholinesterase "in vitro" of three of the more potent piperidine derivatives in comparison with morphine, methadon and meperidine. The cholinesterase activity was measured according to the manometric method of Ammon (12), using a purified cholinesterase from the electric eel, kindly supplied by Dr. D. Nachmansohn of Columbia University. Determinations were usually made in duplicate and a total of 46 measurements were carried out. The average per cent inhibitions are given in table 4, showing that the anticholinesterase activity of the piperidine derivatives is about $\frac{1}{3}$ to $\frac{1}{2}$ of those of morphine and methadon.

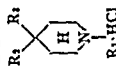

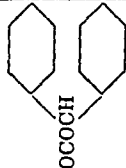

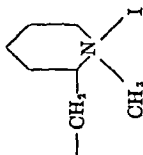


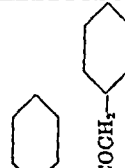





DISCUSSION. In a previous paper from these laboratories (4) it was shown that by reversing the ester group, the analgesic potency of meperidine could be enhanced many fold and that optimal analgesic action is attained with 1-methyl-4-phenyl-4-propionoxy-piperidine. This is in accordance with our findings which demonstrate that any change of these three substituents diminishes or abolishes analgesic potency.

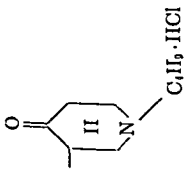
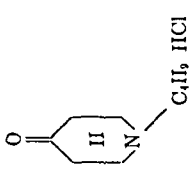






1576	$\begin{array}{c} \text{CH}_3 \\ \diagup \\ \text{CH} \\ \diagdown \\ \text{CH}_3 \end{array}$		H	70	35	18	1/1000	1/150	1/5
1406	$\begin{array}{c} \text{CH}_3 \\ \diagup \\ \text{CH} \\ \diagdown \\ \text{CH}_3 \end{array}$		OCOC ₂ H ₅	40	5 15	51 140	1/1000	1/100	1/2
1333	$\begin{array}{c} \text{CH}_3 \\ \diagup \\ \text{CH} \\ \diagdown \\ \text{CH}_3 \end{array}$		OCOC ₂ H ₅	90	1 3 9	20 43 122	1/1000	1/50	1/2
1190	C ₄ H ₉		OH	350	50	16	1/1000	1/500	1/10
1192	C ₄ H ₉		OCOC ₂ H ₅	400	25	8	1/1000	1/200	1/10
1111	C ₄ H ₉		OH	250	50	0	<1/1000	1/250	1/4
1106	C ₄ H ₉		OCOC ₂ H ₅	200	50	25	<1/1000	1/2000	1/6
1167	C ₄ H ₉		OCOC ₂ H ₅	900	100	10	<1/1000	1/50	1/10
1062	C ₄ H ₉		OCOCH ₃	450	50	10	<1/1000	1/500	1/20

No.				TOXICITY, S.C. Mice LD50	ANALGESIA		SPASMOLYTIC ACTION ON ISOLATED INTESTINE AGAINST			
	R ₁	R ₂	R ₃		s.c. Rats Dose mg./kg.	Percent increase	Acetylcholine (atropine = 1)	Histamine (epine- phrine = 1)	Ba (papav- erine = 1)	
1063	C ₆ H ₅		OCOC ₂ H ₅	350	50	32	1/1000	1/500	1/4	
1105	C ₆ H ₅		OCOC ₂ H ₇	350	50	16	<1/1000	1/500	—	
1066	C ₆ H ₅		OH	500	100	14	<1/1000	<1/1000	—	
1064	C ₆ H ₅		OCOCH ₃	450	50	12	<1/1000	1/250	—	

1065	C_6H_9		$OCOC_2H_5$	450	50	14	$< 1/1000$	1/500	—
1094	C_6H_9		$OCOC_2H_5$	400	100	12			
1307	$CH_3-CH_2-CH(CH_3)-$		$OCOC_2H_5$	135	40	14	1/1000	1/100	1/8
1304			$OCOC_2H_5$	675	25 200	74 140	1/400	1/400	1/2
1077	C_6H_9		$OCOC_2H_5$	175	25 50	42 105	$< 1/1000$		
1056	C_6H_9		$OCOC_2H_5$	150	10 25	49 122	$< 1/1000$	1/1000	1/2

TABLE 3—continued

No.				TOXICITY S.C. MICE LD50	ANALGESIA		SPASMODIC ACTION ON ISOLATED INTESTINE AGAINST		
	R ₁	R ₂	R ₃				Acetylcholine (atropine = 1)	Histamine (epinephrine = 1)	Ba (papaverine = 1)
1907	CH ₃			175 mg./kg.	s.c. Rats Dose mg./kg. 4 16	Percent increase 32 30	1/1000	1/200	>1/5
1186	C ₆ H ₅ , CH ₃ , I	   	OH 	35 mg./kg.	10	28	<1/1000	<1/1000	<1/10
1761	CH ₃	 		85 mg./kg.	2	26	1/400	1/200	1/2
1820	C ₆ H ₅			75 mg./kg.	4 16	20 80	1/1000	1/600	2

1053	C ₄ H ₉		OH	700	100	10	<1/1000	<1/1000	1/250	1/10
1092	C ₄ H ₉		OCOC ₂ H ₅	300	100	18	<1/1000	<1/1000	1/250	1/10
1217	C ₆ H ₁₁		Cl	425	100	10	1/1000	1/1000	1/200	1/5
1254	C ₆ H ₁₁		OC ₂ H ₅	350	100	28	1/500	1/500	1/200	1/2
1280	C ₆ H ₁₁		OC ₃ H ₇	> 100	100	12	1/750	1/750	1/200	1/2
1200	C ₄ H ₉ , CH ₃ I		OCOC ₂ H ₅	175	40	10	1/800	1/800	1/500	1/5
1195	C ₄ H ₉ , O		OCOC ₂ H ₅	150	100	21	1/1000	1/1000	1/250	1/10
1334	CH ₃ , CH ₃ -CH-CH ₃		OCOC ₂ H ₅	100	25 100	88 135	1/1000	1/1000	1/100	1/10

We found, however, that the introduction of alkyl into the 3-position of 1-, 4-, 4-substituted piperidines yields, when tested in rats, the most potent analgesics

jejunum (Thiry-Vella)

↑
Nu-1196
1 mg/Kg, i.v.

↑
Nu-1932
1 mg/Kg, i.v.

FIG. 3. UNANESTHETIZED TRAINED DOG, 22 Kg., THIRY-VELLA FISTULA

Jejunal motility, upstroke = contraction, time in minutes. At arrows, upper record Nu-1196, 1 mg. per kg., lower record, Nu-1932, 1 mg/kg. Injections into saphenous vein. Experiments one week apart.

TABLE 4
Cholinesterase inhibition in vitro

	MOLAR CONCENTRATION						MOL. CONC. TO GIVE 50% INHIBITION
	.0015	.003	.006	.0075	.015	.03	
	Per cent inhibition						
Morphine	16	36	46		65		.006
Methadone	14	22	51		67		.006
Meperidine				5	17	39	>.03
Nu-1196				11	29	68	.02
Nu-1779.				0	19	65	.02
Nu-1932				34	65	93	.01

not derived from morphine. These are the compounds, Nu-1779, Nu-1831, Nu-1832 and Nu-1932. Most striking is the influence of stereoisomerism, best illustrated by comparison of the analgesic potencies of the diastereomers, Nu-1196

and Nu-1779. The potency of the latter, which is 5-6 times that of morphine, is even surpassed by its levo-antipode, Nu-1832. However, substitution in the 3-position has not consistently increased activity; Nu-1333 (table 3) is more potent than the corresponding 3-substituted compound, Nu-1215 (table 1). It seems that the steric arrangement of the substituents in the 3- and 4-positions determines the degree of analgesic potency. According to Lee (13) it is highest when the configuration of the substituents in the 3- and 4-position resembles that

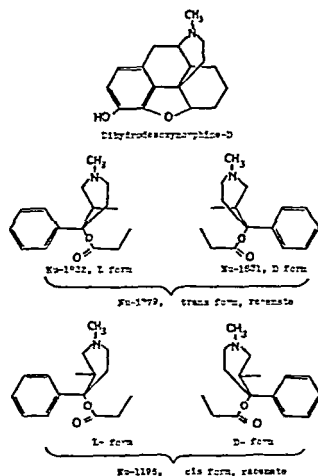


FIG. 4. The assignment of cis and trans configurations is not rigidly established but seems⁸ probable. It is dependent on the easier breakdown of Prep. Nu-1196 under hydrolytic conditions (adjacent hydrogen and propionoxy residues) and upon the physiological results reported here in relation to the known high activity of dihydrodesoxymorphine-D. Similarly the designation of D- and L- for the enantiomorphs, Nu-1831 and Nu-1832, is arbitrary since the optical rotation is too small to be observable. It is here assumed that the more highly active compound, Nu-1832, is more closely related to dihydrodesoxymorphine-D which has a levo rotation (13).

of morphine, as it is apparently the case with Nu-1779 but not with its diastereomer, Nu-1196 (fig. 4).

Qualitatively the more active compounds resemble morphine in their pharmacological action. In adequate doses they produce slowing of the heart rate, salivation and increased intestinal motility presumably due to central vagal stimulation. It seems unlikely that the weak anticholinesterase activity could be an important factor for the intestinal effect in the whole animal.

SUMMARY

A series of 50 derivatives of piperidine have been studied for analgesic action and other pharmacological properties.

Maximal analgesic potency is attained by introducing an alkyl group into the 3-position of these 1-, 4-, 4-substituted piperidines. Steric configuration determines the degree of analgesic potency. Four of these preparations are from four

to eight times as potent as morphine. Qualitatively these compounds resemble morphine in pharmacological action.

Acknowledgment. The technical assistance of Mrs. L. W. Lock, Mrs. A. C. Clark, Miss M. Roe and Mr. P. L. Stefko is gratefully acknowledged.

REFERENCES

- (1) ZIERING, A., BERGER, L., HEINEMAN, S. D., AND LEE, J.: *J. Org. Chem.*, **12**: 894, 1947.
- (2) BERGER, L., ZIERING, A., AND LEE, J.: *Ibid.*, **12**: 904, 1947.
- (3) ZIERING, A., AND LEE, J.: *Ibid.*, **12**: 911, 1947.
- (4) FOSTER, R. H. K., AND CARMAN, A. J.: *THIS JOURNAL*, **91**: 195, 1947.
- (5) DE BEER, E. J.: *THIS JOURNAL*, **85**: 1, 1945.
- (6) HARDY, J. D., WOLFF, H. G., AND GOODELL, H.: *J. Clin. Invest.*, **19**: 649, 1940.
- (7) BLISS, C. I., AND MARKS, H. P.: *Quart. J. Pharm. Pharmacol.*, **12**: 182, 1939.
- (8) WRIGHT, C. I.: *THIS JOURNAL*, **51**: 327, 1934.
- (9) BERNHEIM, F., AND BERNHEIM, M. L. C.: *THIS JOURNAL*, **57**: 427, 1936.
- (10) SLAUGHTER, D., AND LACKEY, R. W.: *Proc. Soc. Exp. Biol. Med.*, **45**: 8, 1940.
- (11) WRIGHT, C. I.: *THIS JOURNAL*, **72**: 45, 1941.
- (12) AMMON, R.: *Arch. ges. Physiol.*, **233**: 486, 1933.
- (13) LEE, J.: *Personal Communication.*

INHIBITION OF SUCCINOXIDASE BY THE VITAMIN P-LIKE FLAVONOID¹ 2',3,4-TRIHYDROXY CHALCONE

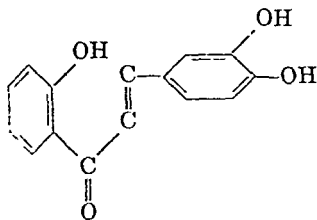
GRANT R. BARTLETT

Scripps Metabolic Clinic, La Jolla, Calif.

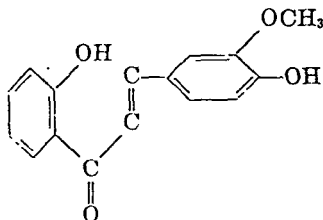
Received for publication March 15, 1948

Certain flavonoid compounds, so-called Vitamin P substances, are of interest pharmacologically in connection with their ability to decrease capillary permeability (1). A postulated mechanism of this action involves the inhibition of epinephrine destruction systems in the body (2). Many of the flavonoids will inhibit epinephrine oxidation *in vitro* and the reaction appears to be associated with either the reducing power or metal chelating capacity of adjacent phenolic groups (catechol structure) (3). One assay technique which is being used to determine the relationship of chemical structure to activity measures the potentiation of epinephrine relaxation of isolated rabbit gut by the vitamin P compounds. Results of the gut assay indicate that the most active compounds possess an orthodihydroxy grouping in conjunction with the basic flavonoid nucleus (4).

In view of the potential therapeutic value of the flavonoids a survey of their influence on certain isolated enzymes is being conducted with the hope that the data may prove useful in explaining and forecasting results *in vivo*. In addition, the problem is of interest to the author because of its bearing on a study being made of the protein-combining abilities of unsaturated ketones (including quinones) and acids. Any ortho- or para-diphenol must be considered as a potential quinone in biological systems. In this study a typical flavonoid containing the catechol grouping, 2',3,4-trihydroxy chalcone (I) and its non-catechol methoxy derivative, 2',4-dihydroxy-3-methoxy chalcone (II) were tested for effects on succinoxidase.



I



II

There are a number of reasons why succinoxidase was chosen for an initial study of flavonoid reactions with enzyme systems. It offers the advantages of ease of preparation and assay and interest due to its widespread occurrence in

¹ Supported in part by a grant from the U. S. Public Health Service.

living cells and its purported importance in metabolic foodstuff degradation. More important, the enzyme contains components which have characteristics of particular interest in respect to possible flavone reactions, namely, an SH-sensitive dehydrogenase, and two oxidase proteins, cytochrome c and cytochrome oxidase which contain metals essential for their activity. Because of the orthodiphenolic nature of the flavones with which we are concerned and the consequent possibilities for oxidation involving quinone formation, there exists the opportunity for reaction with thiol groups of proteins. Succinoxidase and some other proteins in which an intact SH is essential to activity have been shown to be extremely sensitive to traces of quinones (5). On the other hand, the flavones are known to be metal-chelating agents. Thus, it is reasonable to look for special effects on enzyme systems containing metallic components. It is of further importance to determine whether flavone chelation can protect SH-sensitive enzymes from the well known mercaptide type of heavy metal toxicity for which dithiopropanol (BAL) has recently proved to be such a valuable therapeutic reversing agent.

METHODS The 2',3,4 trihydroxy chalcone and its 3 methyl ether were pure yellow crystalline compounds.² Stock solutions were made up in redistilled propylene glycol. The compounds are relatively insoluble in water on the acid side of neutrality. 0.1 cc of a 0.1% solution of the chalcone in propylene glycol added to 2.9 cc of water remains in solution for a few hours and then gradually precipitates. Because of the acidic character of the phenolic groups the compounds are increasingly soluble at higher pH. 0.1 cc of 0.1% solution of the chalcone in propylene glycol added to 2.9 cc of 0.05 M phosphate buffer (pH, 7.6) remains in solution. The free acid is yellow whereas the ion is a very deeply absorbing red.

The succinoxidase preparation was similar to those often used in work with this enzyme (6). The liver from a 200- to 300-gram rat was cooled and minced (in a micro Waring blender) with 40 cc of cold water containing 1.0 cc of 1.0% acetic acid. The mince was filtered through cheesecloth and centrifuged for 10 minutes at 12,000 RPM in a Sorvall conical centrifuge. The supernatant was discarded and the solids resuspended in 40 cc of cold water with the micro Waring. The suspension was centrifuged as before, the supernatant again discarded and the solids made up to a total volume of 40 cc. One half cc of this suspension was used for enzyme activity determinations. The QO_2 with added cytochrome c ranged from 75 to 100 with different rat preparations. The enzyme when stored in the cold retains its activity for 24 hours without significant loss.

The enzyme requires extra cytochrome c in order to attain maximum succinate oxidation and an excess was added in all experiments. Cytochrome c was prepared according to the procedure of Keilin and Hartree (7) except that it was dialysed against distilled water and stored as the lyophilized powder. Its purity was checked by measurement of the absorption of reduced and oxidized forms at 550 $m\mu$ in a DU Beckman Spectrophotometer.

Enzyme activity was determined in standard Warburg equipment, by manometric measurement of the O_2 consumption during the conversion of succinate to fumarate in air at 38°C. Vessels contained 0.5 cc of enzyme suspension, 0.5 cc of 0.5 M phosphate buffers, 0.3 cc of 0.2 M sodium succinate, 0.1 cc of cytochrome c (1.0 mg), 0.2 cc of 15% potassium hydroxide in the center cup, inhibitor and activator solutions, and water to a total volume of 3.2 cc.

² These compounds were supplied by my associate Dr W. G. Clark and had been prepared by Dr T. A. Geissman of the University of California at Los Angeles.

TABLE I

Inhibition of Succinoxidase by 2',3,4-trihydroxychalcone (THC)

ENZYME PREPARATION	pH	TIME INTERVAL	$\mu\text{l. O}_2$ CONSUMED		‰ INHIBITION	CONC. THC (MOLAR)
			Succinate	Succinate-THC		
213	6.8	10-20	80	71	12	1×10^{-4}
216	8.2	10-20	49	25*	49	4×10^{-5}
217	8.2	10-20	42	21†	50	4×10^{-5}
218	8.2	10-20	44	23‡	48	4×10^{-5}
				29§	34	
				15†	40	
		20-30	25	12§	52	
236	6.8	0-10	91	61	33	1×10^{-4}
		10-20	57	21	63	
	7.4	0-10	115	9	92	
		10-20	56	6	89	
	8.2	0-10	41	0	100	
		10-20	30	0	100	
238	6.8	0-10	155	90	42	1×10^{-4}
		10-20	112	74	34	
	7.4	0-10	159	11	93	
		10-20	121	5	96	
	8.2	0-10	14	0	100	
		10-20	10	0	100	
244	7.4	0-10	115	77	33	5×10^{-5}
		10-20	95	65	31	
	8.2	0-10	43	21	51	
		10-20	28	16	43	
245	6.8	0-10	67	55	18	1×10^{-4}
		10-20	62	46	26	
	7.4	0-10	89	55	38	
		10-20	71	30	58	
	8.2	0-10	27	0	100	
		10-20	22	0	100	
247	6.8	0-15	88	48	45	1×10^{-4}
		15-30	62	22	65	
	7.4	0-15	87	0	100	
		15-30	68	0	100	
	8.2	0-15	15	0	100	
		15-30	6	0	100	

* THC added to enzyme 3 mins. before succinate.

† THC and succinate added to enzyme simultaneously.

‡ THC added to enzyme 10 mins. before succinate.

§ THC added to enzyme 5 mins. after succinate.

TABLE I—Continued

ENZYME PREPARATION	pH	TIME INTERVAL	$\mu\text{l. O}_2$ CONSUMED		% INHIBITION	CONC. THC (MOLAR)
			Succinate	Succinate-THC		
250	6.8	0-10	62	36	42	5×10^{-5}
		10-20	53	35	34	
	7.4	0-10	67	13	81	
		10-20	56	7	87	
	8.2	0-10	33	0	100	
		10-20	23	0	100	
251	7.4	0-10	88	19	78	5×10^{-5}
		10-20	55	7	87	
	8.2	0-10	26	0	100	
		10-20	23	0	100	

RESULTS. In Table I are presented data showing the range and potency of the inhibition of succinoxidase by 2',3,4-trihydroxy chalcone (THC) under conditions of varying concentrations, acidities and enzyme preparations. Although there is a considerable variation in the per cent inhibition figures with different preparations, it is readily apparent that THC induces a very strong inhibition of succinate oxidation, an effect which is markedly enhanced with increasing pH. For example, with enzyme #250 the inhibitions produced by $5 \times 10^{-5} M$ THC were 45% at pH 6.8, 81% at pH 7.4 and 100% at pH 8.2.

Substrate in contact with the enzyme before treatment with THC does not protect the protein, as is demonstrated in Table I, preparation #218, where approximately the same degree of toxicity resulted whether the THC was added to the enzyme ten minutes before adding succinate or ten minutes afterwards.

The THC, at a concentration ($1 \times 10^{-4} M$) and pH (8.2) which give complete inhibition of succinoxidase, does not affect cytochrome oxidase activity as measured by catalysis of catechol oxidation with the washed rat liver mince (Table II), thus indicating that the site of action is on the substrate activating dehydrogenase. Experiments to check this point further by using the methylene blue anaerobic technique were unsuccessful because of the formation of an insoluble combination between the dye and the flavonoid.

Glutathione (GSH) was able to prevent completely the succinoxidase inhibition when mixed with the enzyme before addition of THC. However, when the THC was added to the enzyme followed by the introduction of GSH, there resulted no reversal of the inhibition. Apparently the glutathione will combine with THC, abolishing its toxicity, but will not do so once the flavone has reacted with the enzyme.

The 3-methoxy derivative of THC, 2',4-dihydroxy-3-methoxy chalcone, was tested for its effect on succinoxidase at $1 \times 10^{-4} M$ and pH 6.8, 7.4, and 8.2 (See Table IV). There was no inhibition under conditions where the THC is quite toxic. It can be concluded that the diphenolic grouping, a potential quinone-former, plays a part in the inhibition. It is not certain to what extent

THC may form a quinone oxidation product under the conditions of the experiment. There was no oxygen consumption by the THC alone in the presence of

TABLE II
Effect of THC on cytochrome oxidase

VESSEL	O ₂ UPTAKE μl. (0-10 MIN.)	% INHIBITION	CONC. THC
Catechol.....	70	None	1×10^{-4}
Catechol, THC.....	71		

Conditions: 0.5 cc. washed rat liver mince, 0.1 cc. of 0.1% THC in propylene glycol, 0.1 cc. of cytochrome c (2 mg.), 0.3 cc. of 0.1 M catechol, 0.5 cc. of 0.5 M Na₂HPO₄, 0.2 cc. of 20% KOH in center cup, total vol. to 3.2 cc. with water, air, 38° C. Small catechol autooxidation blank subtracted from above figures.

TABLE III
Effect of glutathione (GSH) on THC inhibition of succinoxidase

VESSEL	O ₂ UPTAKE μl. (10-20 MIN)	% INHIBITION
A. GSH added to enzyme 10 min. before THC		
GSH.....	0	None
THC.....	0	
Succinate	60	
Succinate, GSH, THC	60	
B. GSH added to enzyme 10 min. after THC		
Succinate.	44	100
Succinate, THC, GSH	0	

Conditions: succinoxidase assay as described in text

Final concentrations: 1×10^{-4} M THC, 3×10^{-3} M GSH, pH 8.2.

TABLE IV
Effect of the 3-methoxy derivative of THC on succinoxidase

pH	μl O ₂ UPTAKE (10-20 MIN.)		% INHIBITION
	Succinate	Succinate methoxy deriv.	
6.8	87	79	9
7.4	95	95	0
8.2	40	42	0

Conditions: succinoxidase assay as described in text.

Final concentration of 2',3-dihydroxy-4-methoxy chalcone was 1×10^{-4} M.

enzyme. However, because of the small amounts used, an appreciable conversion to quinone could have occurred without manometric evidence. Benzoquinone itself is strongly inhibitory to succinoxidase at concentrations as low as 1×10^{-6} molar.

That THC will combine with cupric ion is indicated by the change in color on mixing the two substances and by the prevention of cupric ion catalysis of epinephrine oxidation. On the other hand, cupric ion is a strong poison of succinoxidase. It was of interest, therefore, to see whether THC would compete with enzyme for combination with cupric ion. THC ($1 \times 10^{-4} M$) was added to the enzyme followed by an equimolar concentration of cupric sulphate. There resulted no prevention of the copper inhibition by THC (Table V). The experiment was carried out at pH 6.7 where there is only a small inhibition by THC but almost complete inhibition by the copper. The evidence suggests that the copper-protein combination is much stronger than is the THC-copper complex.

DISCUSSION. It is believed that the best explanation of the toxic response of succinoxidase to THC is that the latter is partially converted to a quinoid form which inactivates the enzyme by combining with essential sulfhydryl groups. The evidence in favor of this conclusion can be summarized as follows. The inhibitor target is the SH-sensitive succinate activating protein, the cytochrome

TABLE V
Effect of THC on copper inhibition of succinoxidase

VESSEL	O ₂ UPTAKE μ l (0-10 MIN)	% INHIBITION
Succinate	115	
Succinate, Cu ⁺⁺	3	97
Succinate, THC	83	28
Succinate, Cu ⁺⁺ , THC	6	95

Conditions: succinoxidase assay as described in text.

0.08 M final pH 6.7 phosphate buffer, $1 \times 10^{-4} M$ final THC, $1 \times 10^{-4} M$ final Cu⁺⁺ (cupric sulphate). The THC was added to the enzyme 5 min before the Cu⁺⁺.

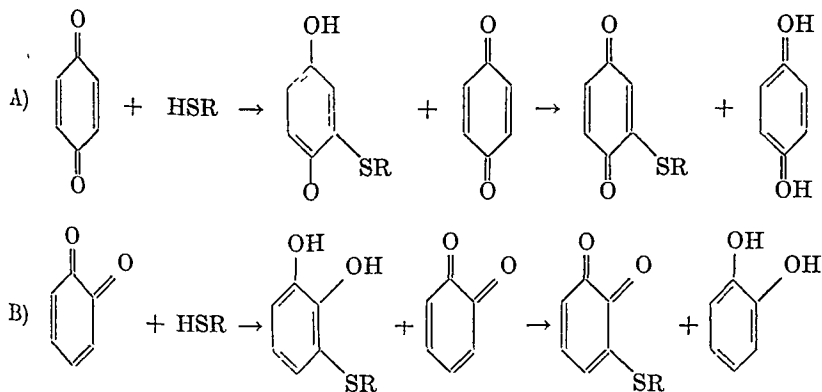
oxidase component being unaffected. The 3-methyl ether of THC, which chemically is unlikely to form a quinoid product under the experimental conditions, does not inhibit the enzyme. One would expect a greater quinone production from THC with increasing alkalinity and under these conditions one finds a greater inhibition of the enzyme. The SH-tripeptide, glutathione, could completely prevent the inhibition, but was unable to reverse it (see discussion below). It is possible that the cytochrome oxidase contributes to the transformation of the chalcone into its quinone since it is known to be a catalyst for the oxidation of catechol.

There is an abundance of data pointing to a selective reaction between protein-SH and quinones. Quastel (8) found that various polyphenols were highly toxic to urease activity and concluded that formation of quinones was responsible. The inhibition could be prevented by thiols but was not reversible. Hellerman and Perkins (9) later discussed the inhibiting effect of quinone on urease and papain. They pointed out the irreversibility of the reaction and suggested an addition of amino or sulfhydryl groups of protein at the reactive olefinic group of the quinone. More recently Potter (10), and Potter and Du Bois (5) have

investigated the inhibition of urease and of succinoxidase by quinones and quinoid products of the oxidation of *p*-aminophenol and *p*-phenylenediamine and their derivatives. They ascribe the inhibition to a reaction with essential thiol groups of these enzymes and conclude that a 1,4-addition of protein-thiol to quinone rather than a direct oxidation by quinone takes place.

A number of workers have described the potent bactericidal action of various quinones. This action has been shown to be prevented by thiols and has been attributed to a combination between quinone and essential thiol constituents of the bacterial cell (11, 12).

A few chemical studies with simple thiols have indicated the type of reaction which may take place between quinone and protein. Snell and Weissberger (13), Kuhn and Beinert (14), and Fieser and Turner (15), have studied the combination of sulfhydryl compounds with quinones. The quinones readily undergo reactions involving the addition of thiol to the 1,4-positions of the α,β -unsaturated ketonic grouping. In general, the course of the reaction would be as follows:



The amino acid cysteine enters into such a reaction with facility under physiological conditions and it is probable that cysteine-proteins will do likewise.

Although it appears from available information that the preferred reaction of a quinone in a competitive mixture of reagents present in a cell extract would be with thiol groupings, nevertheless, it is possible that even at low concentrations other protein reactions will occur. Hoffman-Ostenhof (16) has recently discussed evidence against a specific SH reaction mechanism for explaining quinone toxicity. However a really conclusive answer to the question awaits a careful analysis of the inactivating influence of various quinones on a series of sulfhydryl and non-sulfhydryl enzymes and quantitative titrations with quinones of the various reactive groups of a protein.

Earlier in this paper it was mentioned that substances structurally related to trihydroxy chalcone are metal-complexing substances. The compounds form varied colored soluble and insoluble complexes with many heavy metals. A survey of this field is now in progress by T. A. Geissman at U. C. L. A. In a

qualitative way we have shown that the 2',3,4-trihydroxy chalcone will combine with a number of metals including ferric and cupric ions. It was interesting therefore to note the lack of effect of this compound on the iron-containing cytochrome c and the metalloprotein (presumably Fe), cytochrome oxidase. However, it should be recalled that dithiopropanol (BAL), a potent metal-combining agent, did not react with the metals in these two proteins, but was strongly toxic to some other metalloproteins (17, 18): catalase (Fe), peroxidase (Cu), and carbonic anhydrase (Zn).

It is well known that heavy metals can be highly toxic due to combination with the thiol group of essential proteins. A major contribution of recent years has been the finding that certain simple thiols, notably BAL, are able to reverse the metal mercaptide combination, thus regenerating the activity of the original protein (19). The possibility existed that because of their chelating capacity the flavones might be able to prevent or reverse metal combination with SH-proteins. If so these compounds would be potentially of value in the treatment of metal poisoning. As reported in this paper, at least in the case of copper, the flavone is unable to prevent the metal inhibition of succinoxidase, indicating that the protein-S-copper linkage has a smaller dissociation constant than the copper-flavone-complex.

It is becoming increasingly apparent that the SH function attached to essential protein is a particularly vulnerable item in the animal economy. Heavy metals, certain non-metals such as arsenic and selenium in appropriate valence state, many reactive organic halides, various oxidizing agents, and quinoid structures and the related α,β -unsaturated ketones, all show a marked affinity for protein-SH and as a result can produce profound disturbances in the living organism. In any studies of the physiological effects of the catechol-flavones and other polyphenols, possible quinone formation with resulting blocking of SH-proteins must be kept in mind.

SUMMARY

2',3,4-trihydroxy chalcone gives a strong inhibition of rat liver succinoxidase, an effect which is greater with increasing pH. At a pH of 7.4, 1×10^{-4} M chalcone almost completely inhibits the enzyme.

The chalcone reacts with substrate activating dehydrogenase and not with the cytochrome c-cytochrome oxidase system.

The methoxy derivative, 3-methoxy-2',4-dihydroxy chalcone, is not toxic to the succinoxidase system.

The inhibition is probably due to a combination of quinoid oxidation product of the flavone with essential SH groups of the enzyme. The reaction can be prevented but not reversed by glutathione.

Although the flavone forms complexes with various metals, including copper, it did not decrease the cupric ion inhibition of the succinoxidase.

ACKNOWLEDGMENT

The writer is grateful to Dr. W. G. Clark and Dr. T. A. Geissman for valuable suggestions in connection with this study.

REFERENCES

1. MUNRO, H. N., LAZARUS, S., AND BELL, G. H.: Nutrition Abstr. & Rev., 17: 291, 1947.
2. JAVILLIER, M., AND LAVOLLAY, J.: Helvet. chim. acta, 29: 1283, 1946.
3. LAVOLLAY, J.: I. L'Autoxydation des Diphenols en particulier de L'Adrenaline. II. Structure et Role Fonctionnel de la Vitamine P., Paris, Hermann & Cie, 1943.
4. CLARK, W. G. AND GEISSMAN, T. A.: Federation Proc., 7: 21, 1948.
5. POTTER, V. R. AND DU BOIS, K. P.: J. Gen. Physiol., 26: 391, 1943.
6. ELLIOTT, K. A. C. AND GREIG, M. E.: Biochem. J., 32: 1407, 1938.
7. KEILIN, D. AND HARTREE, E. F.: Biochem. J., 39: 289, 1945.
8. QUAESTEL, J. H.: Biochem. J., 27: 1116, 1933.
9. HELLERMAN, L. AND PERKINS, M. E.: J. Biol. Chem., 107: 241, 1934.
10. POTTER, V. R.: Cancer Research, 2: 688, 1942.
11. COLWELL, C. A. AND MCCALL, M.: Science, 101: 592, 1945.
12. GEIGER, W. B.: Arch. Biochem, 11: 23, 1946.
13. SNELL, J. M. AND WEISSBERGER, A.: J. Am. Chem. Soc., 61: 450, 1939.
14. KUHN, R. AND BEINERT, H.: Ber., 77B: 606, 1944.
15. FIESER, L. F. AND TURNER, R. B., QUOTED IN FIESER, L. F.: Ann. Internal Med., 15: 648, 1941.
16. HOFFMAN-OSTENHOF, O.: Science, 105: 549, 1947.
17. WEBB, E. C. AND VAN HEYNINGEN, R.: Biochem. J., 41: 74, 1947.
18. BARRON, E. S. GUZMAN, MILLER, Z. B., AND MEYER, J.: Biochem. J., 41: 78, 1947.
19. PETERS, R. A., STOCKEN, L. A., AND THOMPSON, R. H. S.: Nature, 156: 616, 1945.
- WATERS, L. L. AND STOCK, CHESTER: Science, 102: 601, 1945.

THE COMPARATIVE PHARMACOLOGY OF THE CYCLOHEXYLALKYLAMINES

DAVID F. MARSH

*Department of Pharmacology, West Virginia University School of
Medicine, Morgantown*

Received for publication March 17, 1948

Since the fundamental study by Barger and Dale (1) of the relationship between the chemical constitution and physiological action of the sympathomimetic amines, numerous investigations have been made of many structurally and pharmacologically related compounds. A few publications have described the pharmacology of compounds in which cyclic arrangements other than the benzene ring have been incorporated into the general sympathomimetic structural pattern (2-5). Zenitz, Macks and Moore (6) have recently synthesized the cyclohexyl analogs of the readily available phenylalkylamines. In this study, 2-phenylethylamine, 2-phenyl-1-propylamine, 1-phenyl-2-propylamine, and their N-methyl homologs have been compared with the corresponding spatially related cyclohexylalkylamines¹

EXPERIMENTAL PROCEDURE *Blood pressure effects in dogs* In order to avoid the error that can be introduced by tachyphylaxis we resorted to a modification of the method of Chen (7) of standardization with an agent, such as epinephrine, that neither produces nor is affected by this phenomenon, and then injecting a test dose of the agent under consideration. Sixty apparently healthy, adult mongrel dogs (4.5-30 kgm) were used, these were anesthetized with sodium barbital (330 mgm/kgm) administered intraperitoneally 90 minutes prior to use. Blood pressure was recorded by the usual mercury manometer and sphygmograph. The animals were standardized with graded doses of epinephrine (1-12 μ gm/kgm) and the 1 mgm test dose of the agent per kgm, as a 1 per cent solution of the hydrochloride salt, was injected into a femoral vein. At 30 minute intervals, this dose of the same drug or a related drug was injected until the blood pressure level permanently changed, or until there was evidence of decreased responsiveness of the animal. The epinephrine equivalence data given in Table 1 is based on the response to the first injection of a drug into the animal after the epinephrine standardization. Five animals were used for each drug.

Action on isolated tissue segments Sections of jejunum from 5 rabbits were placed in oxygenated Tyrode solution at 37-38°C. After some preliminary observations, 10 mgm amine hydrochloride per 50 ml tissue bath was chosen as a standard concentration. After two minutes exposure to the drug, the bath was flushed out three to five times. The responsiveness of the segments to 12.5 μ gm. epinephrine base was used as a control. In a similar manner, virgin albino rat uterine horns in non-oxygenated, glucose free Tyrode solutions

¹ I am grateful to Dr. Jerome Martin of the Commercial Solvents Corporation, Terre Haute, Indiana for the Phenisopropylamine base from which the 1-phenyl-2-propylamine hydrochloride was prepared, to Dr. B. E. Graham of the Upjohn Company, Kalamazoo, Michigan for the "Beta-phenyl-n-propylamine", to Dr. H. W. Werner of the William S. Merrell Company, Cincinnati, Ohio for the "Vonedrine" hydrochloride, and to Dr. M. L. Moore of the Frederick Stearns & Co., Detroit, Michigan for the rest of the sympathomimetic amines. I am grateful to D. A. Herring and C. K. Sleeth for technical assistance.

were exposed to these agents. Sections of ileum of five guinea pigs were suspended in oxygenated Tyrode solution and 5 μ m. histamine base added to the 50 ml. bath. Two minutes later 10 mgm. amine hydrochloride was added and the effects noted after an additional two minutes.








RESULTS. Blood Pressure Effects. In general, the phenyl compounds, or aralkylamines, are more active in raising the blood pressure than are the hydrogenated compounds, or cyclohexylalkylamines. Also, the N-methyl derivatives are usually less active than the unsubstituted amines. The comparison of the blood pressure effects of the phenylethylamines and cyclohexylethylamines is considerably simplified in that four to eight injections can be made at 30-minute intervals without the development of appreciable tachyphylaxis or decrease in response. Thus it is possible to compare two drugs by the procedure of Barger and Dale (1); i. e., to administer one drug between two injections of another drug, and to rule out the possibility that either had any effect on the performance of the other (fig. 1A). They have pointed out that only equimolecular quantities of agents should be compared. The use of a fixed dose of 1 mgm. of agent per kgm. is comparable to the work of others in the field (8-10) and the error introduced is small when the molecular weight range is narrow, as is the case with these agents. However, the data available have been expressed in terms of moles of amine base contained in one mgm. of amine hydrochloride and compared with the pressor equivalent numbers of moles of epinephrine base; the resulting activity ratios are given in the table. Both total weight comparison and molecular comparison lead to the same generalizations.

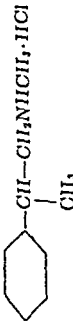
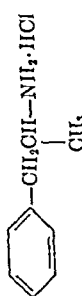
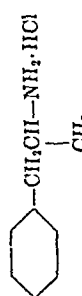
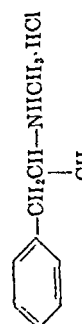
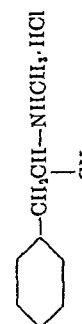
Figure 1A illustrates the greater potency of 2-phenylethylamine over the N-methyl homolog, and 1B its greater activity compared to the cyclohexylethylamine. Similarly, 1C shows the greater activity of 2-phenylethyl methylamine over its analog, cyclohexylethyl methylamine. As indicated in the table, the only appreciable exception to the concept that N-methyl derivatives are less potent than the unsubstituted compounds exists in the case of cyclohexylethyl methylamine and cyclohexylethylamine, with the former usually being more active than the latter (fig. 1D).

2-Substituted-1-propylamines can be administered three to five times at 30-minute intervals without appreciable change in response (fig. 2B). 2-Phenyl-1-propylamine is more potent than 2-cyclohexyl-1-propylamine (fig. 2A); 2-phenyl-1-propyl methylamine is slightly more active than 2-cyclohexyl-1-propyl methylamine (fig. 2C). 2-Phenyl-1-propylamine is slightly more potent (4.8:3.2) than 2-phenyl-1-propyl methylamine, but 2-cyclohexyl-1-propylamine is slightly less potent (2.0:2.2) than 2-cyclohexyl-1-propyl methylamine.

Tachyphylaxis is most pronounced with the 1-substituted-2-propylamines. This phenomenon is well known with 1-phenyl-2-propylamine, or amphetamine, (8, 11), and it occurs with the other agents of this sub-group. This is illustrated in figure 2D. Consequently, direct cross-comparisons are invalid and only the epinephrine equivalence data are useful. 1-Phenyl-2-propylamine is more active than the cyclohexyl compound; 1-phenyl-2-propyl methylamine is more active than its cyclohexyl relative; and the N-methyl homologs are less active than the unsubstituted amines, whether the ring is aromatic or cycloaliphatic.

TABLE I

CHEMICAL NAME AMINE HYDROCHLORIDES	FORMULA	MELTING POINT °C. (UNCORR.)	PRESSOR ACTIVITY		RABBIT JEJUNUM PER CENT CHANGE IN TONE PRO- DUCED BY 200 MG./ LITER	QUINEA FIG- URE CON- TRACTED WITH 100 µG./L DIS- TAMINE PER CENTINNI- TION BY 200 MG./LITER	EAT UTERUS CHANGE IN TONE BY 200 MG./LITER
			Micrograms of epinephrine in pressor equivalent to one mm. amine HCl	Molecules of amine equivalent in pressor effect to one molecule of amine HCl			
2-Phenyl-ethylamine "Phenethylamine"		216-217	10.5	111	+100†	20	++
2-Cyclohexyl-ethylamine		254-256	4.1	274	-50	70	±
2-Phenyl-ethyl methylamine		163-164	7.9	135	+50	40	+
2-Cyclohexyl-ethyl methylamine		171-172	4.5	230	-100†	90	++
2-Phenyl-1-propylamine "Beta-phenyl-n-propylamine"		140-147	4.2	254	+40	40	±
2-Cyclohexyl-1-propylamine		199-200	2.0	510	-100	80	---†
2-Phenyl-1-propyl methylamine "Vonedrine"		144-146	3.8	260	-30	50	-

2-Cyclohexyl-4-propyl methylamine		201-205	2.2	435	-100	100†	—
1-Phenyl-2-propylamine "Amphetamine"		143-145	1.5	237	+50	40	—
1-Cyclohexyl-2-propylamine		191-192	3.1	333	-80	90	—
1-Phenyl-2-propyl methylamine "dl-Desoxyephedrine"		134-135	2.7	365	-10	60	—
1-Cyclohexyl-2 propyl methylamine		127-128	2.0	479	-100	100	—

* Approximately $1-1.3 \times 10^{-3}$ Moles/liter.

† + = increase, - = decrease, ± = both effects noted.

‡ Quantitatively similar effects produced in the same tissue segment by 250 micrograms 1-epinephrine (base) per liter.

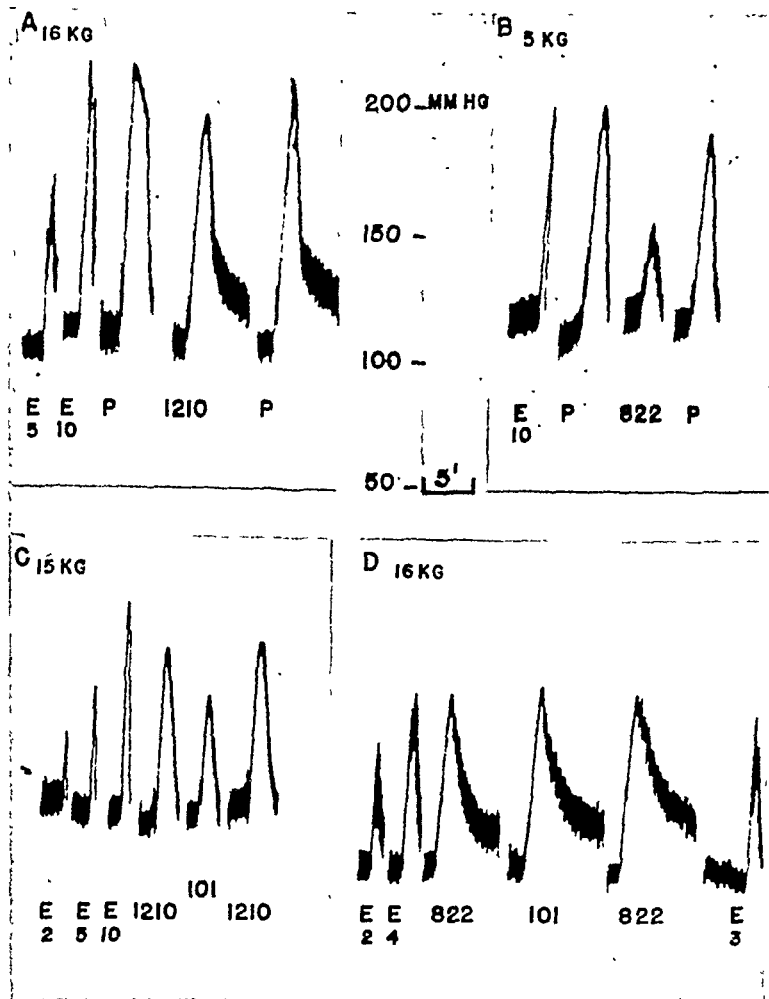


Fig. 1. Dogs. 330 mgm. of Na Barbital per kgm. Blood pressure in mm. Hg, ordinate (Record bottom = 50 mm Hg), time, abscissa. E = 1-epinephrine (base), with dose in micrograms/kgm, time interval between injections, 6-8 minutes. Time interval between injections of other agents, 30 minutes. P = 2-phenylethylamine hydrochloride, 1210 = 2-phenylethyl methylamine hydrochloride, 822 = 2-cyclohexylethylamine hydrochloride, and 101 = 2-cyclohexylethyl methylamine hydrochloride. Doses of these agents, 1 mgm./kgm.

The data on the pressor effects obtained with the various phenylalkylamines are in fair agreement with those of other workers that used similar equivalence assay techniques (8-12). The duration of the rise in blood pressure is difficultly

quantitated. If one uses, as an approximation, the length of time that the blood pressure is above one-half the total increase in pressure, then the effect of the ethylamines lasts one to two times as long as that of epinephrine, the 2-substituted-1-propylamines two to four times as long, and the 1-substituted-2-propylamines three to ten times as long. There is no obvious correlation between

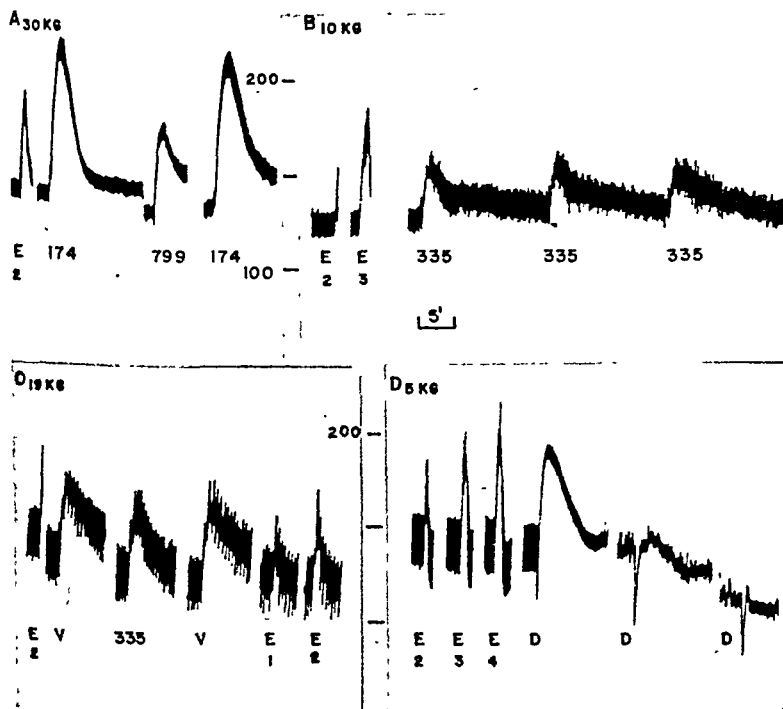


FIG. 2. Dogs. 330 mgm. of Na Barbital per kgm. Blood pressure in mm. Hg, ordinate (Record bottom = 50 mm Hg); time, abscissa E = 1-epinephrine (base), with dose in micrograms/kgm., time interval between injections, 6-8 minutes. Time interval between injections of other agents, 30 minutes. 174 = 2-phenyl-1-propylamine hydrochloride, 799 = 2-cyclohexyl-1-propylamine hydrochloride, 335 = 2-cyclohexyl-1-propyl methylamine hydrochloride, V = 2-phenyl-1-propyl methylamine hydrochloride, and D = 1-phenyl-2-propyl methylamine hydrochloride. Doses of these agents, 1 mgm./kgm.

aromatic and cycloaliphatic compounds, the difference being apparently related to the relative position of the substituent ring in reference to the side-chain length.

Isolated Tissues. The relative importance of data obtained from isolated segments of small animals may be questioned. However, it may provide a closer approximation of the ultimate effects of drugs on cells than does the administration of an agent into a highly integrated system. Quantitatively, the agents are 1/1,000 to 1/10,000 as active as epinephrine or less, rather than 1/100 to 1/500

as is the case with the blood pressure effects. The type of activity is also changed. 2-Phenylethylamine has a more or less indeterminate action on several isolated smooth muscle structures (11, 12). The other members of this series have a more specific action, but one that is apparently not related to the blood pressure effects. The overall trend is an increase in ability to produce relaxation or decrease in tone as the molecular size of the agents increases and as the relative unsaturation decreases; i. e., the cyclohexylalkylamines are more relaxant or tone-inhibitory than the phenylalkylamines, the N-methyl homologs are more active than the unsubstituted amines, and the propyl compounds are more active than the ethyl compounds.

DISCUSSION. Regardless of the mechanism of action of these agents, be it by non-specific "pseudosympathomimetic" effects directly on smooth muscle, and/or by specific "sympathicotropic" effects on the smooth muscle excitatory effectors of the cardiovascular system, or by inhibiting amine oxidase, or by competitively liberating preformed sympathin E at the myoneural junction, the quantitative differences in their activity lead to certain comments. As suggested by Barger and Dale, the phenylethylamine skeleton is necessary for the maximum pressor activity. All other non-hydroxylated agents of similar chemical configuration have a diminished activity. Consequently there must be some specific limitations as to the molecular size and shape of molecules that will have this sympathomimetic effect. Pauling and his co-workers (13) have obtained evidence that the specific forces between an antibody molecule and an antigen are the ordinary forces exerted between smaller molecules that come within a few Angstroms of one another (the van der Waals electronic dispersion forces, the forces of hydrogen bond formation, and the coulomb forces of attraction between groups with electrical charges of opposite sign) and that the specificity of interaction results of a complementariness in structure so that the weak forces operating between the two substances are integrated over the juxtaposed surfaces to produce a strong enough resultant force to lead to the formation of an effective bond. Similarly, one can postulate that the specificity of a sympathetic effector body is the result of certain surface topology with definitely located groups capable of forming hydrogen bonds and one group with a negative electrical charge. Any ammonium ion that has attached to it an organic molecule that does not exceed epinephrine in critical size should have some sympathomimetic action. Minor changes in size, shape, or aromatic character may so change the interaction forces that the resultant bond is ineffective and the expected muscle action does not occur, or occurs in a statistically smaller group of muscles. Conversely, since these agents do not properly fit the effector substances, one may expect that some of them will not interact adequately with the enzyme systems that normally remove or destroy sympathins and consequently they will have a longer duration of action and may even leave the muscle refractory to further effect.

SUMMARY

2-Phenylethylamine, 2-phenyl-1-propylamine, 1-phenyl-2-propylamine and their N-methyl homologs have been compared with their cyclohexyl analogs.

The unsubstituted phenylethylamine is more active as a pressor agent than phenylethyl methylamine, and these are both more active than the corresponding cyclohexylethylamines. Similarly, the phenylpropylamines, though less active than the phenylethylamines, are more active than their cyclohexyl analogs. On isolated smooth muscle, the reverse is true, with the hydrogenated compounds being more relaxant than the aromatic compounds, and the N-methyl homologs being more active than the unsubstituted compounds.

BIBLIOGRAPHY

1. BARGER, G., AND DALE, H. H.: *Jour. Physiol.*, **41**: 19, 1910.
2. TAINTER, M. L.: *Quart. Jour. Pharm. Pharmacol.*, **3**: 584, 1930.
3. ALLES, G. A., AND FEIGEN, G.: *THIS JOURNAL*, **72**: 265, 1941.
4. GUNN, J. A., AND GURD, M. R.: *Jour. Physiol.*, **97**: 453, 1940.
5. LANDS, A. M., LEWIS, J. R., AND NASH, V. L.: *THIS JOURNAL*, **83**: 253, 1945.
6. ZENITZ, B. L., MACKS, E. B., AND MOORE, M. L.: *Jour. Am. Chem. Soc.*, **69**: 1117, 1947.
7. CHEN, K. K.: *THIS JOURNAL*, **33**: 237, 1928.
8. TAINTER, M. L.: *Arch. int. pharmacodyn. et therap.*, **46**: 192, 1933.
9. SWANSON, E. E., SCOTT, C. C., LEE, H. M., AND CHEN, K. K.: *THIS JOURNAL*, **79**: 329, 1943.
10. GRAHAM, B. E., CARTLAND, G. F., AND WOODRUFF, E. H.: *Ind. Eng. Chem., Industrial Ed.*, **37**: 149, 1945.
11. MARSH, D. F., PELLETIER, M. H., AND ROSS, C. A.: *THIS JOURNAL*, **91**: 324, 1947.
12. CHEN, K. K., WU, CHANG-KENG, AND HENRIKSEN, E.: *THIS JOURNAL*, **36**: 363, 1929.
13. PRESSMAN, D., GROSSBERG, A. L., PEIRCE, L. H., AND PAULING, L.: *Jour. Am. Chem. Soc.*, **68**: 250, 1946. (See this article for earlier references.)

THE EFFECTS OF CORN OIL AND OLIVE OIL ON THE BLOOD SUGAR AND RECTAL TEMPERATURE OF RABBITS

E. F. STOILMAN

*Institute of Experimental Biology and Medicine, National Institute of Health,
Bethesda, Md.*

Received for publication March 29, 1948

In the course of work in which DDT was administered to rabbits as a solution in corn oil, it was found that a marked elevation in blood sugar occurred in some animals (1). The administration of corn oil alone also produced hyperglycemia. Since in previous work (2, 3, 4) olive and corn oil had been used extensively as a solvent for DDT administrations, it was decided to study this phenomenon in greater detail.

EXPERIMENTAL PROCEDURE. Normal rabbits of the Dutch strain inbred at this laboratory were used exclusively throughout these experiments. They were placed in individual metabolism cages and maintained on a standard diet of oats and cabbage except where otherwise indicated.

The oils¹ were administered by mouth, uniformly followed by 10 cc. per kilo water. The animals were bled from the ear vein just preceding the administration of the oil and at regular intervals thereafter. Four cc. of blood constituted the usual sample. A few milligrams of sodium oxalate were used as an anticoagulant. The bloods were deproteinized by the method of Folin and Wu (5). Determinations for blood sugar were made by the method of Schales and Schales (6). Figures submitted by these authors indicate that from 15-24 per cent of the total reducing substances obtained with the Folin-Wu filtrate of normal human blood are non-fermentable, and do not represent true glucose.

All determinations were made in duplicate on the same blood filtrate. All readings agreed within the limits of experimental error, and the results in each case represent the average of the two analyses. The results were evaluated in terms of glucose. Rectal temperatures were taken immediately preceding the bleedings.

Effect of Administration of Oils on Blood Sugar. Corn oil or olive oil given in amounts of 3-15 cc. per kilo produced hyperglycemia. This is shown in table 1. On the larger doses of either olive oil or corn oil the animals became ill and refused food and water for several days until either death or recovery occurred. A set of controls were therefore run under these conditions, namely without food or water. The withdrawal of food and water had little effect on the blood sugar during the first 48 hours. During the following 48 hours there was a moderate progressive elevation. However, the blood sugar had returned to slightly above the normal level by the end of the sixth day. A second set of controls on the regular diet maintained a normal uniform blood sugar level. Table 2.

Effect of Administration of Oils on Rectal Temperature. The animals in each of the groups, which received either corn oil or olive oil experienced a drop in

¹ Clinton refined corn salad oil, purchased from Clinton Industries incorporated. Two brands of olive oil were used. One labeled pure and packaged by Philip Berio and C. Lucca, Italy; and one labeled Pompeian imported pure olive oil.

rectal temperature. In general hypothermia and hyperglycemia occurred simultaneously. (Table 3)

TABLE 1

The effect of oral administration of oil on the blood sugar in rabbits

No & Sex	WEIGHT—KG		BLOOD GLUCOSE MG %—HOURS AFTER ADMINISTRATION													
	Initial	Final	0	2	4	7	11	24	48	72	96	120	144	168	192	
(15 cc Per Kilo Corn Oil)																
1♀	2 24	1 99	116 5	135 5	142 0	141 3	158 3	459 0	D							
2♀	2 52		109 3	118 0	142 5	160 0	186 3	D								
3♀	1 86	1 35	123 0	138 3	198 3	180 5	198 5	344 0	233 0	193 5				D		
4♀	2 01	1 81	150 5	146 8	176 0	180 3		208 8	168 5	168 5				142 5		
5♀	2 80	2 36	134 5	170 8	200 0	252 5		210 0	272 5	D						
6♂	2 30	2 12	135 8	194 3	217 0	201 0		218 5	183 0	161 0				127 3		
(15 cc Per Kilo Olive Oil)																
7♀	1 96	1 54	129 8	142 5	146 3	141 4		182 5	277 0	276 5	D					
8♀	1 71	1 54	135 0	174 5	214 5	199 5		277 0	206 0	153 5			134 5		145 8	
9♀	1 55	1 24	114 5	145 0	185 4	180 3		205 5	222 0	192 0		D				
10♀	2 53	2 17	125 0	157 5	161 5	173 0		147 5	137 0	127 5	130 0			141 5		
11♀	2 59	2 18	132 0	157 8	165 0	176 5		202 0	220 0	151 5	158 0			156 5		
12♀	1 93	1 71	131 8	145 0	138 0	142 5		167 0	448 0	D						
(6 cc Per Kilo Corn Oil)																
13♀	1 72	1 46	137 5	157 0	175 8	196 0		350 0	200 5		329 0	D				
14♀	1 75	1 59	120 0	130 5	134 3	140 3		148 5	146 0	134 0						
15♀	2 14	2 04	152 5	155 0	152 3	174 4		239 5	177 8	160 3						
16♀	1 89	1 70	138 5	156 3	174 5	318 0		140 9	134 5							
17♀	2 19	1 99	137 3	148 8	164 5	167 5		162 0	183 0							
(3 cc Per Kilo Corn Oil)																
18♀	1 90	1 88	148 0	147 0	178 5	172 5		183 5	159 5	155 0						
19♂	2 20	2 06	168 5	154 0	177 0	173 0		182 5	168 5	174 5						
20♂	2 11	1 94	130 0	128 5	157 5	149 0		146 5	142 0	150 0						
21♀	2 01	1 90	154 5	219 0	163 5	173 0		155 5	143 0							
22♂	2 20	2 04	139 5	173 0	255 0	412 0		189 0	188 5							
23♀	2 05	1 94	103 5	111 9	108 4	116 9		133 0								
24♀	1 93	1 85	109 6	125 2	140 9	137 3		130 0								
(3 cc Per Kilo Olive Oil)																
25♀	2 27	2 23	110 0	124 8	152 8	148 0		152 9	138 5			140 5				
26♀	2 35	2 37	122 3	135 0	133 0	160 3		150 5	152 0			164 0				
27♂	1 56	1 50	138 0	143 3	134 8	147 3		158 0	153 0			159 5				
28♂	1 68	1 59	150 0	142 0	149 5	148 0		169 0	178 0	160 5						
29♂	1 87	1 89	167 5	166 5	195 0	167 5		173 5	165 0	171 0						
30♀	2 63	2 55	148 0	221 0	354 0	197 5		132 5	141 5	141 5						

D = Approximate time of death

Toxicity of Oils The data are too limited to supply other than indications of the toxic potencies of the two oils. Of the six animals having received 15 cc per kilo corn oil, all had symptoms of toxicity. Four of these died in $5\frac{1}{2}$ –6 $\frac{1}{2}$ days. Of the four one was negative on postmortem. Three had congested lungs in varying

degrees, and two recovered. Of the six animals receiving 15 cc. per kilo olive oil, all had symptoms of toxicity. Three of these died in 2½-5 days. Two had congested lungs. Aspiration pneumonia secondary to administration of the oils by stomach tube may have been a factor in the production of the lung pathology.

In the total series of 45 animals, having received either one of the two oils, or water by stomach tube, only eight animals died. In addition to the seven animals which died on large doses of oil, one of the animals having received 6 cc. per kilo corn oil died. All animals in this group had symptoms of toxicity in less degree than those having received 15 cc. per kilo of either corn oil or olive oil. The animals in the two groups having received the small non-fatal doses of oils recovered after symptoms of toxicity which were in general proportional to the

TABLE 2
The effect of oral administration of water on the blood sugar in rabbits

NO. & SEX	WEIGHT—KG.		BLOOD GLUCOSE REDUCTION EQUIVALENT MG. %—HOURS AFTER ADMINISTRATION												
	Initial	Final	0	2	4	7	21	48	72	96	120	144	168	192	
(25 cc. Per Kilo Water)															
31 ♀	2.24	2.10	117.3	136.3	125.0	128.5	146.3	133.5							
32 ♀	2.02	1.95	131.3	153.3	123.5	128.8	133.5	135.0							
33 ♀	2.00	1.91	132.3	155.8	138.8	141.8	152.3	170.0	154.3	128.5		127.5			
34 ♀	2.56	2.60	133.8	136.0	137.5	136.0	142.5	133.8	126.0	128.3		121.3			
35 ♂	2.23	2.21	124.0	138.5	138.0	139.5	145.0	157.5	137.5	127.0		136.0			
36 ♂	1.79	1.72	140.3	144.3	130.0	129.5	133.5	129.8		145.3	132.8				
37 ♂	1.74	1.60	142.0	138.8	136.0	144.5	145.5	140.3		137.0	141.3				
38 ♀	2.04	1.94	140.0	165.8	143.3	129.3	139.3	128.8		137.3	134.3				
(30 cc. per kilo water—Thereafter no food nor water)															
39 ♂	1.97	1.27	150.0			154.5	151.8	143.3	156.3			168.5	186.0	192.8	
40 ♂	2.77	1.52	117.3			117.3	120.0	133.5	141.6			129.3	127.0	134.3	
41 ♂	2.21	1.41	124.8			117.3	111.8	125.8	135.3			139.0	142.8	152.0	
42 ♀	1.92	0.92	150.5			135.3	123.8	160.5	202.0			151.3	168.5	158.8	
43 ♀	2.56	1.69	115.3	134.3	127.3	119.5	109.0	147.3	147.8	163.3			145.3	161.3	
44 ♀	2.66	1.58	128.0	131.3	136.2	137.8	162.3	154.5	186.3	188.5			148.0	156.8	
45 ♀	2.22	1.44	151.8	159.0	167.0	151.8	136.5	146.8	167.3	198.3			184.3	172.5	

size of the dose received. In contrast, the animals on the standard diet gave no indications of abnormality of any kind. The control fasting group lost considerable weight.

DISCUSSION. In general the hyperglycemia, and the simultaneous hypothermia were proportional to the size of the dose of oil administered. The wide variations within a group having received the same dose probably indicates the wide variation as to the amount and time of absorption of the particular oil from the intestine after its administration. It was previously shown that when DDT was administered orally in olive oil to rabbits there was a marked variation in the amount of DDT excreted in the feces (4). This variability in intestinal absorption may be the primary reason for the relatively wide range in the lethal dose of DDT in rabbits, when the DDT is administered as an olive oil solution.

TABLE 3

The effect of oral administration of oil on rectal temperature in rabbits. Animal numbers correspond to those in Table 1

No.	RECTAL TEMPERATURES DEGREES CENTIGRADE—HOURS AFTER ADMINISTRATION												
	0	2	4	7	11	24	48	72	96	120	144	168	192
(15 cc. per kilo corn oil)													
1	39.3	39.2	38.7	38.7	38.2	38.0D							
2	39.3	39.3	38.9	38.8	38.5D								
3	39.6	39.3	38.7	38.5	38.3	37.8	38.5	38.8			37.6D		
4	39.2	37.9	37.9	37.7		37.4	38.0	38.7				38.4	
5	39.2	38.7	37.4	37.6		38.5	38.3	D					
6	39.0	39.1	38.9	38.4		38.8	38.5	38.6				39.0	
(15 cc. per kilo olive oil)													
7	39.7	39.2	39.5	39.7		38.8	37.9	37.3	D				
8	39.9	38.6	38.2	38.1		38.1	38.8	39.2			39.3		39.3
9	39.3	38.9	38.7	38.7		38.8	38.6	38.7		D			
10	39.4	38.7	38.6	38.5		38.8	39.4	39.7	39.8			39.4	
11	39.5	38.8	38.8	38.9		38.5	38.6	38.9	39.4			39.6	
12	39.9	39.9	39.9	39.5		38.3	37.4D						
(6 cc. per kilo corn oil)													
13	39.8	39.3	39.0	39.2		38.4	39.0	39.0	38.5D				
14	39.3	39.2	39.2	38.9		38.9	39.2	39.4					
15	39.2	38.8	39.0	38.9		38.5	39.4	39.5					
16	39.4	38.7	38.8	38.2		39.3	39.2						
17	39.7	39.8	39.3	39.0		38.9	39.7						
(3 cc. per kilo corn oil)													
18	39.2	39.1	39.3	39.2		39.3	39.2	39.4					
19	39.4	38.6	38.4	38.8		39.1	39.2	39.2					
20	39.5	38.9	39.1	39.4		39.3	39.3	39.3					
21	39.1	37.9	38.8	39.1		38.8	39.5						
22	39.8	39.2	38.7	39.1		39.0	39.8						
23	39.3	39.2	39.3	39.1		39.5							
24	39.6	38.8	39.2	38.7		39.2							
(3 cc. per kilo olive oil)													
25	39.6	39.4	39.9	39.6		39.5	39.4			39.5			
26	39.8	39.6	39.6	39.4		39.0	40.1			39.6			
27	39.7	39.6	39.4	38.9		39.0	39.1			39.7			
28	39.9		39.7	39.9		39.5	39.8	40.1					
29	39.9		39.6	39.4		39.5	39.5	39.5					
30	39.7		38.4	38.8		39.6	39.5	39.6					

D—Approximate time of death.

Blixenkrone-Møller (7) found that the perfusion of blood containing butyric acid through cats' livers led not only to the production of ketone bodies, but also to the production of carbohydrate. Markees and Reich (8) found that butyric, caproic, caprylic and capric acids in the form of their Na salts after oral administration to fasting rabbits raised the blood sugar level. Nath and Brahmachari (9) showed that hyperglycemia could be produced in rabbits by the injection of the intermediary fat metabolism products, β -hydroxy-butyric acid, acetoacetic acid and pyruvic acid.

In several experiments in which corn oil was administered in 15 cc. per kilo, and olive oil in 9-15 cc. per kilo doses by mouth, and 18-24 hours later when the blood sugar was at a very high level 3-5 units of insulin were administered subcutaneously, the elevated blood sugars produced by the oils were promptly reduced to normal or subnormal levels.

Salant and Bengis (10) found that feeding of olive oil to rabbits (one experiment) was followed by renal disturbance and transitory albuminuria. Whether kidney damage was a factor in the causation of death in the animals having received 15 cc. per kilo of either olive, or corn oil in the present experiments has not been determined.

SUMMARY AND CONCLUSIONS

1. Administration of either corn oil or olive oil produced in rabbits hyperglycemia and hypothermia.
2. The elevations in blood sugar and drop in rectal temperature with relatively large doses of corn oil occurred earlier than with equivalent doses of olive oil.
3. Both corn oil and olive oil when administered in relatively large doses appear to be toxic.

REFERENCES

1. STOHLMAN, E. F. AND LILLIE, R. D.: *THIS JOURNAL*, **93**: 351, 1948.
2. SMITH, M. I. AND STOHLMAN, E. F.: *Pub. Health Rep.*, **59**: 979, 1944.
3. NELSON, ARTHUR A., DRAIZE, JOHN H., WOODWARD, GEOFFREY, FITZHUGH, O. GARTH, SMITH, JR., BLACKWELL, R., AND CALVERY, HERBERT O.: *Pub. Health Rep.*, **59**: 1009, 1944.
4. SMITH, M. I. AND STOHLMAN, E. F.: *Pub. Health Rep.*, **60**: 283, 1945.
5. FOLIN, OTTO AND WU, H. SIEN: *Jour. Biol. Chem.*, **38**: 81, 1919.
6. SCHALES, OTTO AND SCHALES, SELMA S.: *Archives of Biochemistry*, **8**: 285, 1945.
7. BLIXENKRONE-MØLLER, N.: *Zeitschr. für Physiol. Chem.*, **252**: 137, 1938.
8. MARKEES, S. AND REICH, I.: *Schweiz. Med. Wochenschr.*, **70**: 185, 1940.
9. NATH, M. C. AND BRAHMACHARI, H. D.: *Nature*, **154**: 487, 1944.
10. SALANT, W. AND BENGIS, R.: *THIS JOURNAL*, **9**: 529, 1917.

THE EFFECT OF DDT ON THE BLOOD SUGAR AND OF GLUCOSE ADMINISTRATION ON THE ACUTE AND CHRONIC POISONING OF DDT IN RABBITS

E. F. STOHLMAN AND R. D. LILLIE

*Institute of Experimental Biology and Medicine, National Institute of Health,
Bethesda, Maryland*

Received for publication March 29, 1948

INTRODUCTION

Following the administration of DDT to rabbits by mouth as a solution in olive oil, stimulation of the central nervous system after a latent period produces involuntary body tremors and general muscular contractions which in some instances continue uninterrupted for several hours with great severity (1, 3). It would seem that under these conditions a great amount of both nervous and muscular energy would be required and expended by the animal organism, until a state of exhaustion, prostration and collapse is reached. This is likely to create a great demand on the glycogen reserves in the liver and muscles.

It was decided to determine: 1) what effect DDT would have on the blood sugar, 2) the effect glucose administration would have on the symptoms and the mortality due to DDT poisoning, also whether glucose would afford some protection to the vital organs against the pathological effects of DDT poisoning.

METHODS The experimental procedures used in this work are in essence an extension of those used in the preceding paper (8). Except where otherwise indicated the methods are identical.

ACUTE TOXICITY STUDIES **The Effect of Oral Administration of DDT on the Blood Sugar and Rectal Temperature** As shown in the preceding paper, corn oil when administered to rabbits elevates the normal blood sugar level and produces a simultaneous drop in rectal temperature (8). When DDT was administered in a well tolerated dose (300 mg per kilo), in a relatively small amount of corn oil, an elevation in blood sugar and a simultaneous drop in rectal temperature approximated that produced by the administration of an equivalent amount of corn oil alone (8). However, when DDT was administered in a relatively large dose, 600 mg per kilo in a moderate amount of corn oil, producing severe symptoms and death within 18 hours, the degree of blood sugar elevation attained (table 1) was substantially greater than that produced by the administration of an equivalent amount of corn oil alone. Further, the elevation in blood sugar produced under these conditions was more acute in point of onset, and returned to a near normal level by the time the blood sugar reached its maximum following the administration of an equivalent amount of corn oil without DDT (8).

The Effect of Intravenous Administration of DDT on the Blood Sugar. Using a modification of a method previously used for intravenous DDT administration (4), DDT was injected intravenously to rabbits as a solution in Tween 20, except in one instance in which it was administered as a finely divided colloidal suspension in Tween 20 in water. This animal received 20 mg per kilo DDT as a 0.5% suspension in a 7% solution of Tween 20 in water. Another animal was given the

same dose but as a 5.4% solution of DDT in undiluted Tween 20; a third animal received 30 mg. per kilo DDT as a 5.4% solution. Two additional animals received 30 mg. per kilo DDT as an 8% solution. Four controls received equivalent amounts of Tween 20 as were given to the animals receiving DDT.

By this method of administration DDT produced a pronounced elevation in blood sugar. In some instances the increases in blood sugar were 100-200% of

TABLE 1

The effect of acute DDT poisoning on the blood sugar in rabbits. DDT administered as a solution in corn oil per os

NO. & SEX	WEIGHT—KG.		HOURS AFTER ADMINISTRATION OF DDT, MG. %										
	Initial	Final	0	2	4	7	11	13.5	15	18	24	48	96
(300 Mg. per kilo DDT as a 5% solution)													
1♂	1.70	1.66	116.5							155.0		115.0	110.0
2♀	2.23	2.36	119.5							115.0		116.3	120.0
3♂	1.74	1.61	117.5							128.0		121.3	90.0
4♂	2.23	2.19	123.8							131.3		137.5	115.5
5♀	1.54	1.48	115.0							125.5		122.5	121.0
(300 Mg. per kilo DDT as a 10% solution)													
6♀	1.95	1.85	153.0	173.0	215.3	228.3					151.5		
7♀	2.06	1.95	165.5	159.8	170.5	175.5					160.8	140.3	
8♀	2.13	2.07	107.4	120.7	122.5	128.5					113.5		
9♀	2.01	1.87	110.0	132.5	159.5	164.8					118.3	131.3	
(600 Mg. per kilo DDT as a 10% solution)													
10♂	2.27		125.8	219.0	190.0	198.0					170.0*		
11♀	2.10	1.90	112.8	108.3	97.5	120.0					142.8		125.5
12♂	1.84	1.60	93.0	105.8	138.8	140.0					147.0	122.0	116.8
13♀	2.60		95.5	116.3	160.0	165.0					79.5†		
14♂	1.88	1.70	113.0	118.3	168.0	178.8					147.0	118.3	123.3
15♂	1.82		110.0				224.0	395.0					
16♂	2.00	1.89	117.8				110.5	122.5		127.8			123.3
17♂	3.17		121.3				227.0	232.8	175.3				

* Blood sugar at 26 hours just preceding death 142.5.

† Blood sugar at 30 hours, 7 hours preceding death 93.3.

the normal. The change occurred in 10-30 minutes following the injection and a maximum elevation was reached in from 1-2 hours.

If the animal survived the effects of a single administration for two or more hours, the blood sugar returned to a normal level with the recovery of the animal. However, in one instance in which it dropped to a subnormal level the animal died (fig. 1, curve 1). It should be pointed out, however, that the vehicle Tween 20, in which the DDT was administered also produced some elevation in blood sugar (fig. 1, curves 6-9).

By repeated administration of 15 mg. per kilo DDT at about 2 hour intervals, very high blood sugar levels of 266.9-581.0 mg. per cent were obtained.

Under the same conditions repeated injections of equivalent amounts of the vehicle Tween 20 had no appreciable effect on the blood sugar.

The Effect of Glucose Administration Following the Oral Administration of DDT in Corn Oil. In the following experiments 600 mg. per kilo DDT as a 10% solution in corn oil was administered to both experimental and control animals. One gm. per kilo glucose was administered as a 10% solution in distilled water intravenously to the experimental animals, immediately following DDT administration, and as the severity of the symptoms warranted further injections of

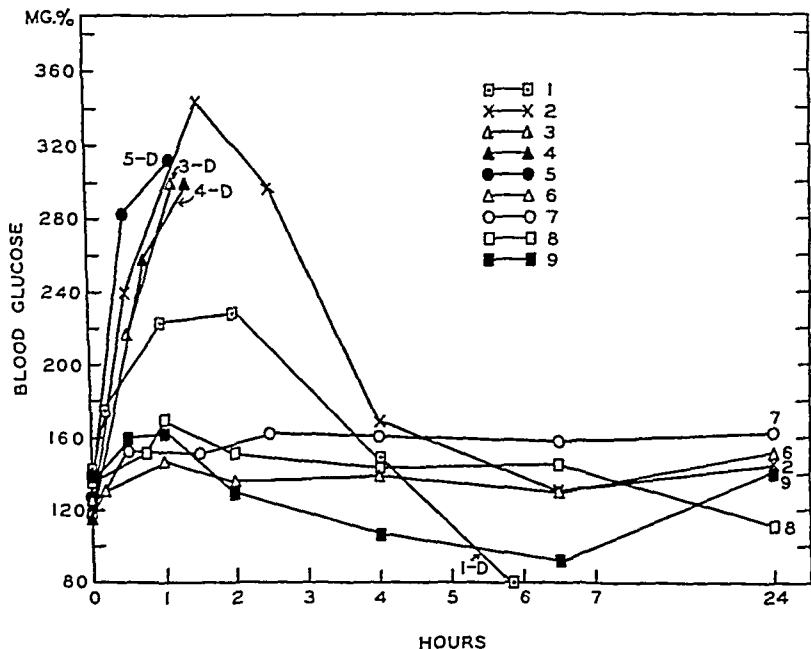


FIG. 1. Effect of intravenous DDT on blood sugar (D indicates death of animal).
 1, 2. 20 mg/kg DDT.
 3, 4, 5. 30 mg/kg DDT.
 6. Control, 4 cc/kg 7% aqueous solution of Tween 20.
 7, 8, 9. Controls, 0.38 cc/kg undiluted Tween 20.

glucose were given at 18, 42 and 66 hours thereafter. After the initial administration of glucose, subsequent administrations were made immediately following bleedings for blood sugar analyses. In some cases an additional administration of glucose was given between blood sugar analyses.

The results of these experiments are shown in table 2. The administration of glucose had practically no effect on the DDT hyperglycemia at 18 hours and thereafter. At this time the maximum blood sugar elevation had already passed, and the blood sugar was well on the way to normal.

Though very high levels of blood sugar can be obtained by the administration of glucose intravenously (fig. 2), they are not maintained for any appreciable

length of time. However, administrations of this character in some instance may serve to replenish a deficit in glycogen reserves.

The Effect of Intravenous Administration of DDT in Tween 20 on the Rectal Temperature. In all the animals to which DDT was administered intravenously rectal temperatures were taken immediately preceding bleedings for blood sugar determinations. When fatal, or relatively large doses (20-30 mg. per kilo) of DDT were administered intravenously there was in general an initial elevation

TABLE 2

The effect of intravenous glucose administered as a 10% solution in distilled water on the blood sugar in rabbits following the administration of DDT in corn oil per os

NO & SEX	WEIGHT—KILOS		1 GM./KIL O GLUCOSE							BLOOD SUGAR—MG %			
	Initial	Final	Hours after DDT admin							Hours after DDT admin			
			0	18	24	42	48	66	0	18	42	66	
(600 mg per kilo DDT as a 10% solution plus glucose)													
18 ♀	1 86	1.71	1	1	1					133 0	117 0	155 0	160 0
19 ♀	1 62	1 38	1	1	1	1		1	1	116 9	157 5	130.0	
20 ♀	1 85	1 57	1	1	1	1				120 0	164 0	135.0	
21 ♀	3 15	2 66	1	1	1	1		1	1	135 8	242 0	119 0	158 0
22 ♀	2 07	1 82	1	1	1					115 8	127 3	117 5	
23 ♀	1 88	1 56	1	1						135 8	171 0		
24 ♂	1 54	1 42	1	1						152 3	166 0		168 0
25 ♂	2 23	2 04	1	1						119 3	149 0		143 0
26 ♂	1 65	1 53	1	1						106 3	168 0		145 8
27 ♂	1 7		1							113 8			
(600 mg per kilo DDT as a 10% solution without glucose)													
28 ♀	1.90									119 5			176 8
29 ♀	1.60	1 34								99 3	118 0	135 5	
30 ♀	1 93	1 80								137 5	164 5	140 0	
31 ♀	3 30									127 5	250 0		
32 ♀	1 77	1 55								119 5	151 5	140 0	
33 ♀	2 22	1 75								150 0	189 0		
34 ♂	2 00	1 88								139 0	154 3		130 0
35 ♂	1 82	1 74								121 0	190 8		118 3
36 ♂	1 92	1 64								111 0	133 3		135 0
37 ♂	1.74	1 60								128 8	127 8		107 3

in rectal temperature which rose parallel with the hyperglycemia, except in one instance in which it dropped to a subnormal level. In contrast, moderately large doses of Tween 20 (0.38 cc. per kilo) caused a slight drop in rectal temperature (figure 3). The actual elevation in temperature produced by DDT would probably be greater than indicated by the readings recorded, if it were not lessened and modified by the effect of Tween 20, which appears to lower the temperature.

The effects on rectal temperature following repeated intravenous administration of 15 mg. per kilo DDT at 2 hour intervals were more variable than they

were on the blood sugar. In one animal there was a moderate elevation in temperature, while in two animals there was a progressive drop at the time when the blood sugar was well above normal in all three.

The Effect of Glucose Administration on the Rectal Temperature Following the Oral Administration of DDT in Corn Oil. Glucose had no appreciable effect on the drop in rectal temperature at 18 hours and thereafter following the administration of 600 mg. per kilo DDT as a 10% solution in corn oil. This is shown in figure 4, curves 2 and 3.

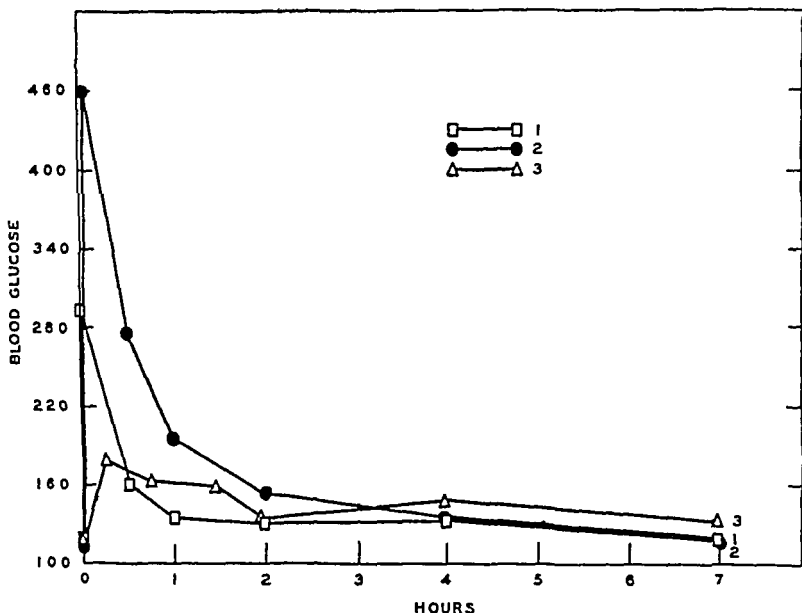


FIG. 2. Effect of parenteral administration of glucose on blood sugar.

Average of 2 animals.

1. 0.55 gm/kg intravenously.

2. 1.0 gm/kg intravenously.

3. 1.1 gm/kg intraperitoneally.

The Degree of Severity of Objective Symptoms Produced by the Oral Administration of DDT in Corn Oil. In these experiments, in which the acute poisoning following the administration of DDT alone, and in the experiment in which glucose was administered in acute DDT poisoning, the following classification was arbitrarily used as a basis of evaluating the degree in severity of the symptoms:—No observation; 0, negative; + hyperexcitable; 2+ mild general tremors; 3+ moderate tremors; 4+ severe; 5+ very severe, including convulsions.

There was considerable variation in the time of onset of symptoms as well as in the degree in severity following DDT administration in corn oil. In general

the degree in severity of symptoms produced by DDT is proportional to the size of the dose of DDT administered (fig. 5, Curves 1 and 2).

Objective Symptoms Produced by the Intravenous Administration of DDT in Tween 20. While in general the symptoms produced by the administration of DDT by mouth are identical with those produced by intravenous administration, in the latter they were quick in onset and rapidly progressive as compared to a lag of 3-6 hours with considerably slower and more gradual progression following

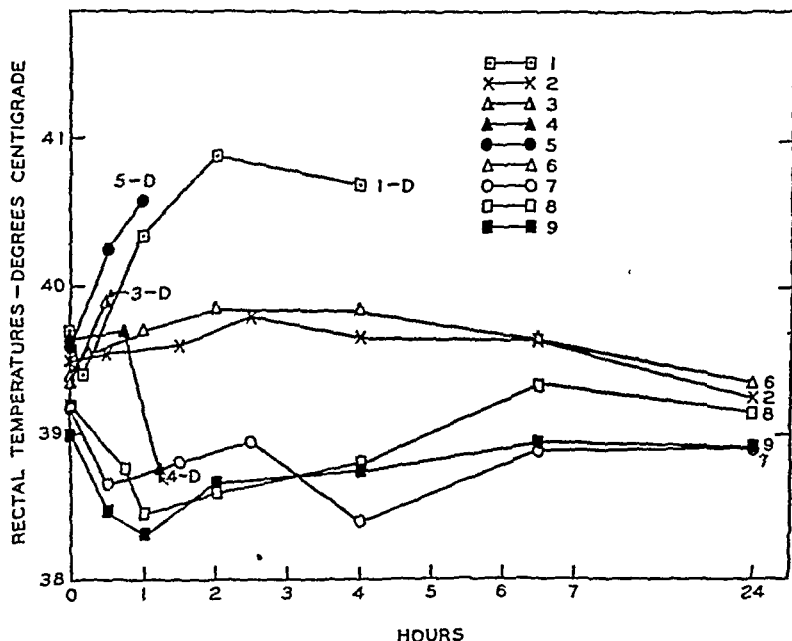


Fig. 3. Effect of intravenous DDT on rectal temperature (D indicates death of animal).
 1, 2. 20 mg/kg DDT.
 3, 4, 5. 30 mg/kg DDT.
 6. Control, 4 cc/kg 7% aqueous solution of Tween 20.
 7, 8, 9. Controls, 0.38 cc/kg undiluted Tween 20.

oral administration. In some instances, hyperexcitability, twitching of the eyelids and mild tremors about the head occurred before the intravenous administration of DDT had been completed. From this stage progression to moderate general tremors with accelerated respiratory rate was a matter of from 10-30 minutes. This was followed by severe tremors and convulsions when a 20 mg. per kilo dose had been administered. With 30 mg. per kilo doses this cycle was accelerated and convulsions sometimes occurred within 10 minutes following the completion of the injection, with recurring convulsive episodes at intervals of from 5-30 minutes. The convulsions lasted about 2 minutes. In some instances

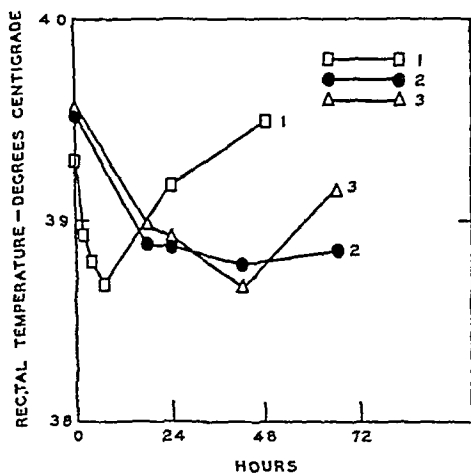


FIG. 4. Effect of oral DDT on rectal temperature Average of 4-10 animals.

1. 300 mg/kg DDT.
2. 600 mg/kg DDT and glucose intravenously.
3. 600 mg/kg DDT, no glucose

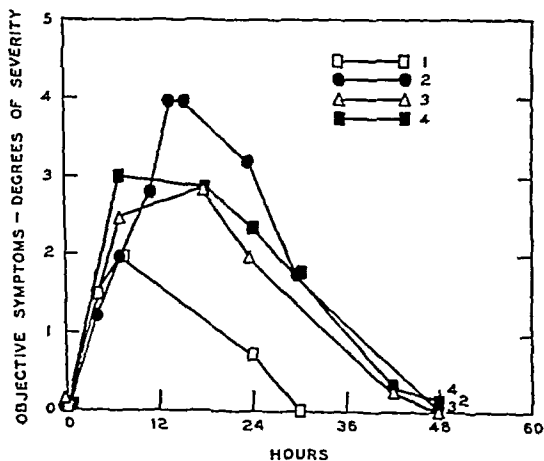


FIG. 5. Symptomatology as related to dosage, in oral DDT poisoning, rated on the basis of 0-5 (see text).

1. 300 mg/kg DDT, average of 4 animals.
2. 600 mg/kg DDT, average of 8 animals.
3. 600 mg/kg DDT and glucose intravenously, average of 10 animals.
4. 600 mg/kg DDT, average of 10 animals

the animal died during the convulsive seizure. In other instances the convulsions were followed by a state of depression with a slowing of the respiratory rate, and diminution in the severity of the general tremors. While the depression was

at first moderate, it usually became progressive until death of the animal from what appeared to be central respiratory paralysis. During the 10-40 minute period of progressive depression the blood sugar remained high. In cases of recovery the elevated blood sugar progressively dropped as the severity of the symptoms diminished.

The Effect of Glucose Administration on the Symptoms Produced by the Oral Administration of DDT in Corn Oil. In animals receiving a single dose of 600 mg. per kilo DDT, there was no discernible difference in the character or in the degree in severity of symptoms, between those animals having received glucose and those not having received glucose (fig. 5, Curves 3 and 4).

Mortality Produced by the Oral Administration of DDT in Corn Oil. Of 9 animals which had received 300 mg. per kilo DDT, all recovered and subsequently gained weight, after varying degrees of symptoms. Four out of 8 animals, which had received 600 mg. per kilo DDT died, two of them within 15 hours, and the other two in 26-37 hours. This group suffered a 50% mortality. All animals were weighed at regular intervals. In each group there was considerable variation from animal to animal as regards weight loss and time of occurrence (1-10 days) following DDT administration.

The Effect of Glucose Administration on the Mortality Produced by the Oral Administration of DDT. Three out of each group of 10 animals in this experiment died. In the group which received glucose one died within 18 hours; two in 5-6½ days. Both of the latter developed flaccid paralysis within 18-24 hours after DDT administration, which continued until death. In the control group two animals died within 18½-22 hours, and the third in 3½ days. This animal developed flaccid paralysis 18 hours after DDT administration which persisted until death. Since there was a 30% mortality in each group glucose administrations had no apparent effect on survival.

CHRONIC TOXICITY STUDIES. DDT was administered as a 5% solution in olive oil in 150 mg. per kilo doses three times a week to a mixed group of 6 male and female rabbits until death of the animals. Glucose was administered orally as a 5% solution in water, intravenously as a 10% solution, or intraperitoneally as a 5.4% solution five days a week at 6, 24, and 30 hours following each administration of DDT.

During the experimental period this group received an average of 14 doses of DDT, an average of 14 doses of 1 gm. per kilo glucose by mouth, an average of 13.6 doses of 1 gm. per kilo glucose intravenously, and an average of 23.8 doses of 1 gm. per kilo glucose intraperitoneally.

A mixed control group of 9 male and female rabbits received an average of 15.6 doses of DDT, without any other administrations.

The treated animals lived an average of 33.8 days as compared with the control animals, which lived an average of 37.4 days. The treated animals lost an average of 410 gms., as compared to an average loss of 220 gms. for the control group.

Objective Symptoms Produced by Repeated Administrations of DDT in Olive Oil. The symptoms of DDT intoxication varied in severity in each individual animal throughout the course of the experiment, from no symptoms to symptoms of

varying intensity. On the basis of the maximum degree of severity of symptoms attained by each animal, some time throughout the experimental period, the maximum average degree in severity was 2.6 for the treated group as compared to 3.3 for the control group.

The Effect of Administration of Glucose on the Pathogenesis Produced by Chronic DDT Poisoning. In addition to 5 of the glucose treated rabbits in the previous experiment, 5 additional rabbits were given repeated administrations of DDT in olive oil by mouth and glucose intravenously. Three of the latter received 150 mg. per kilo DDT, two received 200 mg. per kilo DDT and from 1-5 gms. per kilo glucose intravenously per dose.

Similarly, in addition to the 9 untreated rabbits in the control group 9 additional rabbits were given repeated administrations of DDT in olive oil by mouth. Three of these received 150 mg. per kilo DDT and 6 received 200 mg. per kilo DDT per dose.

The average daily intake of DDT in animals surviving over 10 days was 75.3 mg. per kilo in the treated series. The total glucose intake varied from 9 to about 88 gms. The average daily intake of DDT was 63.8 mg. per kilo in the untreated series. The survival periods averaged 27.3 days for the treated series and 45.8 for the untreated.

Necrosis, often extensive, was present in the livers of 14 of the 18 rabbits in the untreated series, and in 3 of 10 of the glucose treated series. The central cytoplasmic hyaline oxyphil alteration of liver cells described in previous papers (2, 3, 5) appeared in the livers of 12 of the 18 untreated animals and in 4 of the 10 treated. Fatty degeneration of variable grade was present in all of the livers of the untreated series and in only 7 of the treated animals. Besides the lower incidence in the treated series the fatty changes were also less extensive and less severe. Glycogen (Bauer reaction) was absent in 15 of the 16 livers in the untreated series. Glycogen was demonstrated in small to moderately large amounts in 8 of the 10 livers in the treated series. In general glycogen tended to be absent in those livers in which necrosis and the cytoplasmic hyaline degeneration were most pronounced.

Fatty degeneration of the renal convoluted or loop tubules, or both was present in 17 of 18 rabbits in the untreated series and in 5 of 10 in the glucose treated series. Congestion and edema of the lung appeared in 12 untreated and in 5 treated rabbits. Fatty degeneration of heart muscle was noted in 17 untreated and 4 treated animals, and was further apparently more severe in the untreated group.

Hemosiderosis of the spleen pulp was absent in 3 treated and 2 untreated rabbits, slight in 1 treated and 2 untreated, moderate in 2 treated and 8 untreated, and severe in 2 treated and 5 untreated rabbits; an average grade on a 0-4 basis of 1.5 in the treated and 1.9 in the untreated series.

The adrenals were studied in nearly all the animals and presented no significant lesions. The pancreas was studied in a few, with similarly negative findings.

Pyogenic pneumonia or purulent bronchitis was present in 5 rabbits, 3 treated and 2 untreated.

The glucose treatment apparently decreased the extent and frequency of le-

sions in the organs studied. This apparent decrease in lesions, however, was not associated with a longer survival time or increased tolerance to the poison.

Discussion. Little can be said about the mechanism by which DDT produces an elevation in blood sugar and an elevation of rectal temperature in rabbits. Its elucidation awaits further work. It may be suggested that the DDT in the adrenal glands may in some manner stimulate this tissue to liberate epinephrin, which in turn would elevate the blood sugar. It is well known that injection of epinephrin produces hyperglycemic effects in animals (6). It is also possible that both these effects may be of central nervous origin.

On the other hand the hyperglycemia, and hyperthermia may be a physiological response of the organism to meet a critical need for increased energy in the form of glucose, which may be rapidly consumed by the muscles in their involuntary contractions produced by DDT stimulation of the central nervous system, and as soon as the crisis abates the blood sugar and temperature return to normal levels.

In acute toxicity experiments in which DDT was administered intravenously to cats in a soyalecithin-corn oil emulsion Koster (7) found that glucose given before or immediately after an LD_{50} dose of DDT reduced convulsions and mortality. If the glucose was given preceding the administration of DDT, it also reduced the severity of the tremors. However, glucose was ineffective against an LD_{50} dose of DDT except to increase the survival time. In the present experiments, glucose was ineffective in reducing the severity of the symptoms in acute DDT poisoning when DDT was administered by mouth in corn oil. In chronic experiments in which repeated doses of DDT in olive oil were given, glucose administrations did not reduce the average loss in body weight, nor did it increase the average survival time. However, it appeared to decrease the severity of the symptoms and the pathologic effects on some of the viscera.

The negative effect of glucose on the symptoms and survival in the acute poisoning and the length of survival in chronic oral poisoning of DDT, may be due to the availability of additional blood sugar resulting from: 1) the elevation in blood sugar produced by the DDT oil solvent (8) in which the DDT was administered, and 2) the mobilization of sugar (perhaps from body glycogen stores) produced by DDT itself.

Further work is required to learn something of the mechanism by which glucose apparently decreased the extent and frequency of pathological lesions in the organs studied.

SUMMARY AND CONCLUSIONS

1. Acute severe DDT poisoning, resulting from the administration of DDT in corn oil, produced an elevation in blood sugar in excess of that produced by the administration of an equivalent amount of corn oil alone.

2. With the administration of large doses of DDT in corn oil, the elevation in blood sugar occurred several hours earlier than with the administration of equivalent amounts of corn oil alone, and returned to normal levels considerably sooner than with corn oil.

3. With the administration of a well tolerated dose of DDT (300 mg. per kilo) in corn oil, the maximum drop in rectal temperature occurred at the time of the maximum elevation in blood sugar. The elevation in blood sugar and drop in rectal temperature obtained with this dose were essentially due to the corn oil and not the DDT.

4. In general when DDT was administered by mouth the maximum severity of the symptoms occurred at the time of the maximum elevation of blood sugar and the maximum drop in temperature.

5. Intravenous administration of DDT in Tween 20 produced a hyperglycemia and a hyperthermia.

6. The administration of glucose did not reduce the average loss in body weight, nor the average mortality in acute poisoning with DDT in corn oil.

7. The administration of glucose did not reduce the average loss in body weight, nor did it increase the average survival time in chronic DDT poisoning, in which DDT was administered in olive oil. It did appear to ameliorate somewhat the severity of the symptoms, and apparently decreased the extent and frequency of lesions in some of the tissues.

REFERENCES

1. SMITH, M. I. AND STOHLMAN, E. F.: Pub. Health Rep., 59: 984, 1944.
2. LILLIE, R. D., AND SMITH, M. I.: Pub. Health Rep., 59: 979, 1944.
3. NELSON, ARTHUR A., DRAIZE, JOHN H., WOODWARD, GEOFFREY, FITZHUGH, O. GARTH, SMITH, JR., BLACKWELL, R., AND CALVERY, HERBERT O.: Pub. Health Rep., 59: 1009, 1944.
4. SMITH, M. I., BAUER, HUGO, STOHLMAN, E. F. AND LILLIE, R. D.: THIS JOURNAL, 88: 359, 1946.
5. LILLIE, R. D., SMITH, M. I. AND STOHLMAN, E. F.: Arch. Path., 43: 127, 1947.
6. SOLLMAN, T.: A Manual of Pharmacology, Sixth Edition, 1942.
7. KOSTER, RUDOLF. Federation Proceedings, 6: 346, 1947.
8. STOHLMAN, E. F.: THIS JOURNAL, 93: 346, 1948.

THE TOXICITY AND MUSCULAR EFFECT OF d-TUBOCURARINE COMBINED WITH β -ERYTHROIDINE, MYANESIN OR EVIPAL*

F. M. BERGER, M.D. AND R. P. SCHWARTZ, M.D.

The Department of Pediatrics and The Department of Surgery, Division of Orthopedics, The University of Rochester School of Medicine and Dentistry, Rochester 7, New York

Received for publication March 31, 1948

It has been shown that curare can relieve muscular spasm of varied origin (1-4). The narrow margin between effective doses and those causing systemic effects seriously limits the usefulness of the drug (5). The experiments presented in this paper were carried out to discover whether it would be possible by the simultaneous administration of other agents to increase the margin of safety and usefulness of the mixture.

The drugs administered jointly with d-tubocurarine were β -erythroidine, myanesin and evipal. β -Erythroidine was chosen because it has a similar action on the myoneural junction as d-tubocurarine, but a different action on the central nervous system (5, 6). Because of the possibility of influencing the peripheral action of d-tubocurarine by the simultaneous use of central depressants, myanesin and evipal were used. Myanesin does not influence the myoneural mechanisms in tolerated doses, but has a depressant effect on the spinal cord (7, 8). With larger doses an ascending depression of the motor pathways of the central nervous system is obtained. Certain pharmacological and clinical experiments indicate that myanesin may have a selective depressant action on the nuclei of the midbrain (9, 10). It has been used clinically as a substitute for curare during anesthesia (11). Evipal is a typical short acting barbiturate causing a descending paralysis of the central nervous system. It has been frequently used in conjunction with curare during anesthesia.

EXPERIMENTAL TECHNIQUE. All experiments were carried out on white, male mice weighing 14 to 20 grams. The drugs were injected intraperitoneally according to the body weight of the animals.

The influence of drugs or mixtures of drugs on muscle power was estimated by the rotating cylinder technique described by Young and Lewis (12) for the mouse assay of insulin and applied by Skinner and Young (13) for the assay of curare. The animals were placed into the rotating cylinder immediately after injection and mice falling away from the cylinder during 20 minutes were considered as reactors. With lethal doses, the number of deaths occurring 24 hours after injection was counted.

Groups of 10 to 40 mice were injected with graded doses of the drugs or mixtures of drugs and the mean effective or lethal dose and its standard error evaluated according to the method of Miller and Tainter (14). The slope of the dosage mortality line was calculated according to Lichtfield and Fertig's (15) formula.

RESULTS AND DISCUSSION. Table I gives the mean effective and lethal doses, their standard errors, and the slopes of the dosage-effect lines of the drugs when administered individually.

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

It is of interest to note that d-tubocurarine had the steepest dosage-effect line of the four drugs. The slope of the dosage-mortality line of d-tubocurarine was, however, quite flat, indicating that the response of the animals to muscular effects of d-tubocurarine was much more uniform than their response to the lethal action of the drug.

The experimental design for examining the joint action of drugs was similar to that proposed by Bliss (16). Mixtures of d-tubocurarine with one of the other drugs were prepared in proportions of 1:3, 2:2 and 3:1 in terms of their mean effective (ED_{50}) and mean lethal doses (LD_{50}). Graded doses of each mixture were injected to groups of mice and dosage-effect curves constructed. The mean doses of the various mixtures were found graphically and expressed in terms of d-tubocurarine.

The combined action of two drugs administered jointly may be classified in several groups: (1) Independent action occurs when the action of one drug is not

TABLE I

Mean effective and mean lethal doses of d-tubocurarine, β -erythroidine, myanesin and Evipal on intraperitoneal administration to male white mice

	$LD_{50} \pm SE^*$	b*	$ED_{50} \pm SE$	b
d-Tubocurarine	0.5 \pm 0.034	9.0	0.2 \pm 0.009	18.0
β -Erythroidine	24.0 \pm 0.93	17.3	13.8 \pm 0.96	13.1
Myanesin	600.0 \pm 22.4	20.7	92.0 \pm 6.7	10.7
Evipal	280.0 \pm 20.4	12.5	28.0 \pm 1.8	14.4

LD_{50} Mean lethal dose in mg/kg

ED_{50} Mean effective dose in mg/kg

SE Standard error

b Slope.

markedly influenced by the presence of the other. When the curves for the two constituents differ in slope, one may expect an abrupt break in the dosage-effect curve of the mixture (16). The two rectilinear segments above and below the break would be expected to have a similar slope as the original constituents (2). Additive action may be complete when the combined administration of two drugs in complementing proportions of their equitoxic doses causes a similar effect as an equitoxic dose of either constituent administered alone. The two constituents behave as if they were the same substance (substitutive addition of Loewe, (17)). Incomplete addition occurs when the combined effects of the two drugs is greater than that of each constituent administered alone, but smaller than that expected on the basis of arithmetical summation. This type of synergism has been called hetero addition by Loewe (17). (3) Potentiation occurs when the combined effect of the mixture is greater than would be expected from simple addition.

The type of synergism between the action of two drugs can be illustrated graphically by plotting equitoxic doses of various mixtures of the drugs in terms of the more potent constituent against the concentration of the other constitu-

ents of the mixture. In Figure 1, the line OA represents the mean lethal dose of curare in the absence of other drugs and the line OB the mean lethal doses of the other drugs when given alone. If an effect produced by the combination of two drugs lies on the line AB, the drugs have a complete additive action. If the effect of the combined action of two drugs is represented by points inside the triangle AOB, potentiation is taking place, and if it is represented by points above the line AB incomplete addition is spoken of. In the case of independent and different action the points would lie near a line drawn through point A parallel to OB.

The mean lethal and mean effective doses and their standard errors expressed in terms of d-tubocurarine are given in Table II. The slopes of the individual dosage-effect lines and the deviation of the observed mean dose expressed as a percentage of the dose expected to give an additive effect is also given. The results are also illustrated in Figure I.

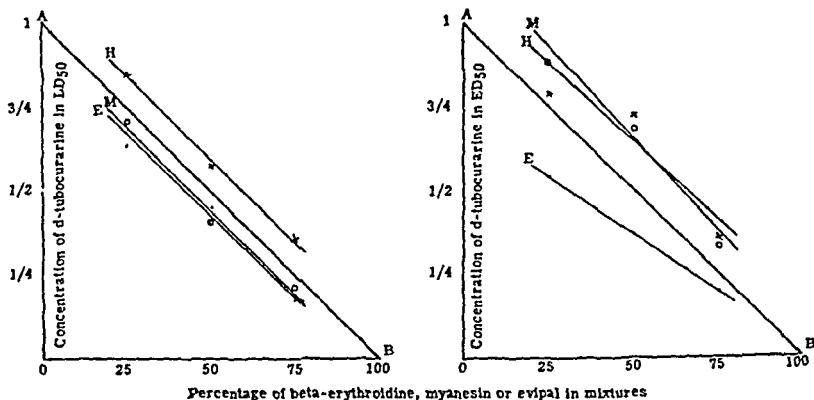


FIG. 1. The mean effective and mean lethal doses of mixtures of d-tubocurarine with β -erythroidine, myanesin or evipal, plotted in terms of d-tubocurarine against the concentration of the other constituent in the mixtures.

When lethal doses were given, the combined effect of d-tubocurarine and β -erythroidine or myanesin was potentiated. The joint action of d-tubocurarine and evipal was incompletely additive. The effect obtained with the three mixtures of each pair of drugs appeared to be largely independent from the ratio of one component to the other. When the LD_{50} doses of the mixtures were plotted against their percentage compositions straight lines parallel with summation line AB were obtained.

When the effect of the combinations was judged by the muscular weakness as measured by the inability of the animals to maintain themselves on the rotating cylinder different results were obtained. The joint effectiveness of d-tubocurarine and β -erythroidine was potentiated to a greater extent than their lethal action. The ED_{50} doses of the mixtures plotted against their percentage composition again gave a straight line which was, however, flatter than the summation line

AB indicating that over the range of combinations examined potentiation increased in proportion to the d-tubocurarine contents.

d-Tubocurarine administered with myanesin or evipal were less effective than would have been expected on the basis of simple summation of effects. The ED_{50} dose plotted against their percentage composition gave curves showing an upward convexity, indicating that summation was least when the mixtures consisted of equal parts of each constituent. This relation was particularly marked in the case of evipal.

TABLE II

Mean effective and lethal doses after combined administration of d-tubocurarine and β -erythroidine, myanesin or evipal on intraperitoneal administration to male white mice

Doses in mg. per kg. body weight expressed in terms of d-tubocurarine

RATIO OF DRUGS	$LD_{50} \pm SE^*$	b*	D*	$ED_{50} \pm SE$	b	D
1 T* + 3 E*	0.096 ± 0.006	10.4	-23	0.037 ± 0.003	6.5	-26
2 T + 2 E	0.222 ± 0.011	15.6	-11	0.071 ± 0.005	5.5	-29
3 T + 1 E	0.315 ± 0.022	12.6	-16	0.107 ± 0.007	8.2	-29
1 T + 3 M*	0.104 ± 0.0048	23.4	-17	0.065 ± 0.006	9.2	+30
2 T + 2 M	0.205 ± 0.014	22.5	-18	0.134 ± 0.008	14.5	+34
3 T + 1 M	0.35 ± 0.014	22.3	-7	0.173 ± 0.016	7.9	+15
1 T + 3 H*	0.172 ± 0.014	9.4	+38	0.071 ± 0.009	5.6	+42
2 T + 2 H	0.282 ± 0.014	14.0	+13	0.143 ± 0.02	3.1	+43
3 T + 1 H	0.42 ± 0.014	16.6	+12	0.157 ± 0.016	5.2	+5

T d-Tubocurarine.

E β -Erythroidine.

M Myanesin.

H Evipal.

LD_{50} Mean Lethal Dose in mg./kg.

ED_{50} Mean Effective Dose in mg./kg.

SE Standard error.

b Slope of the dosage-effect line.

D Percent deviation from dose giving additive effect expressed in terms of d-tubocurarine.

In order to ascertain whether the combined administration of d-tubocurarine and β -erythroidine would be more advantageous and safer than d-tubocurarine given alone, it was necessary to take into consideration not only the potentiated effect, but also the relative safety of such a combination. The so called "therapeutic index" of a drug or combination of drugs is often given as a ratio of the mean lethal dose to the mean effective dose. Expressed in this way the values for d-tubocurarine and β -erythroidine administered alone were 2.5 and 1.74. The ratios of the mean lethal to the mean effective dose of the three mixtures of these drugs gave somewhat higher values, namely 2.6, 3.1 and 2.9. Different results were, however, obtained when due attention was given to the slopes of

the dosage-mortality and dosage-effect lines. Foster (18), taking this into account, proposed the use of the standard safety margin which gives the percentage above the surely effective dose (ED_{99}) at which an occasional death will occur (LD_1) according to the formula $\left(\frac{LD_1}{ED_{99}} - 1\right) 100$. d-Tubocurarine given alone had a standard safety margin of 3%. Mixtures of d-tubocurarine and β -erythroidine had no margin of safety. It would, therefore, appear that the joint administration of the two drugs would be less safe than an equally effective dose of d-tubocurarine given alone. It is, however, possible that some of the side effects of both drugs may cancel each other out on joint administration in man. There is no evidence of this in mice. All animals receiving a mean effective dose of d-tubocurarine showed little side effects apart from a partial loss of muscular strength and coordination as evidenced by their inability to maintain themselves on the cylinder. Animals receiving a mean effective dose of d-tubocurarine and β -erythroidine on the other hand appeared somewhat hyperexcitable, and 3 out of 40 of those injected with the mixture containing the drugs in the proportion of 2:2 developed tonic convulsions and died.

The joint lethal action of d-tubocurarine and myanesin was potentiated, but there was only incomplete summation of the effects as judged by the ability of the animals to maintain themselves on the rotating cylinder. These observations are in agreement with the findings (8) that myanesin does not possess curare-like action in the small doses affecting voluntary muscles. The slight degree of synergism observed between d-tubocurarine and myanesin differs from the potentiation apparent after joint administration of myanesin and evipal (8).

In view of the frequent use of d-tubocurarine during barbiturate anesthesia the joint effect of the two drugs is of interest. Under the conditions of these experiments the joint effect of d-tubocurarine and evipal resulted in incompletely additive action. The dosage-effect lines of the combinations were very flat and there was no standard margin of safety between lethal and effective doses. Although excellent results have been obtained by the use of curare during anesthesia, some anesthetists believe that the procedure is risky and decreases the safety of anesthesia. The experiments described in this paper appear to support this contention.

The results of this investigation indicate that there is little likelihood of increasing the usefulness of d-tubocurarine by the simultaneous administration of other drugs. The margin of safety of d-tubocurarine administered alone is small enough and joint administration of three other drugs with widely differing modes of action reduced this margin still further. In the opinion of the authors the real advance in the treatment of spastic and dystonic states awaits the discovery of drugs possessing a selective depressant action on specified levels of the central nervous system.

SUMMARY

d-Tubocurarine and β -erythroidine administered jointly show potentiation in effective and lethal doses. The margin of safety of the mixture of the drugs is

smaller than that of d-tubocurarine administered alone. The combined administration of d-tubocurarine and myanesin showed slight potentiation in lethal doses and incomplete additive action in effective doses. d-Tubocurarine and evipal showed incomplete additive action in both effective and lethal doses. The significance of the findings is discussed.

ACKNOWLEDGMENTS. Our thanks are due to Dr. C. H. Hodge for the loan of his rotating cylinder apparatus and to Mr. D. E. Leary for technical assistance.

REFERENCES

1. WEST, R.: *Proc. Roy. Soc. Med.*, **25**: 1107, 1932.
2. BENNETT, A. E.: *Am. J. Med. Sc.*, **202**: 102, 1941.
3. DENHOFF, E. AND BRADLEY, C.: *New England J. Med.*, **226**: 411, 1942.
4. SCHLESINGER, E. B.: *Arch. Neurol & Psychiat.*, **55**: 530, 1946.
5. HARVEY, A. M. AND MASLAND, R. S.: *THIS JOURNAL*, **73**: 304, 1941.
6. BURMAN, M. S.: *THIS JOURNAL*, **69**: 143, 1940.
7. BERGER, F. M. AND BRADLEY, W.: *Lancet* *i*, 97, 1947.
8. BERGER, F. M. AND BRADLEY, W.: *Brit. J. Pharmacol.*, **1**: 265, 1946.
9. BERGER, F. M.: *Brit. J. Pharmacol.*, **2**: 241, 1947.
10. STEPHEN, C. R. AND CHANDY, J.: *Canad. Med. Ass. J.*, **57**: 463, 1947.
11. MALLINSON, F. B.: *Lancet* *i*, 98, 1947.
12. YOUNG, D. M. AND LEWIS, A. H.: *Science*, **105**: 368, 1947.
13. SKINNER, H. G. AND YOUNG, D. M.: *THIS JOURNAL*, **91**: 144, 1947.
14. MILLER, L. C. AND TAINTER, M. L.: *Proc. Soc. Exper. Biol. & Med.*, **57**: 261, 1944.
15. LICHTFIELD, J. T. AND FERTIG, J. W.: *Bull. Johns Hopkins Hosp.*, **69**: 276, 1941.
16. BLISS, C. I.: *Ann. Appl. Biol.*, **26**: 585, 1939.
17. LOEWE, S.: *Ergebn d. Physiol.*, **27**: 47, 1928.
18. FOSTER, R. H. K.: *THIS JOURNAL*, **65**: 1, 1939.

THE ABSORPTION, DISTRIBUTION, EXCRETION AND FATE OF PARA-AMINOSALICYLIC ACID¹

E. LEONG WAY, PAUL K. SMITH, DONALD L. HOWIE, ROWENA WEISS
AND ROLLAN SWANSON

*Department of Pharmacology, The George Washington University School of
Medicine, Washington, D. C.*

Received for publication May 3, 1948

p-Aminosalicylic acid (PASA) has been reported to be of experimental (2, 5, 6, 8) and of clinical value (1, 3, 4, 7) in the treatment of tuberculosis. Inasmuch as our laboratory has been investigating the fate of p-aminobenzoic acid and salicylic acid (9, 10, 11, 12) we became similarly interested in PASA due to its close structural relationship to these two compounds.

Investigations have been made on the blood levels obtained with various dosages of PASA and on the excretion of PASA (1, 2, 7, 21, 22). Lehman (1, 2) and Alan and Difs (7) accounted for approximately thirty-five percent of the total dose in human urine. Other investigators (21, 22), in studies on rabbits, reported recovery of seventy to ninety percent of the administered dose in urine, with a large fraction being present as acetylated PASA. Further studies on its fate seemed indicated and are the basis of this report.

PROCEDURES AND RESULTS

Determination of PASA. PASA was determined spectrophotometrically by using established color reactions for aromatic amines and phenols. Solutions of the compound deteriorate rapidly on standing at room temperature but stability can be enhanced by addition of sodium hydroxide or hydrochloric acid.

Modification of the Bratton-Marshall method (13) permitted the determination of PASA satisfactorily, the chief changes being to effect diazotization of an acid solution of the amine at 0-10° and to then couple the mixture with the dye in the presence of acetic acid. The maximum intensity of the color thus developed appeared within ten minutes and did not change materially on standing at room temperature for two hours. Inasmuch as this method measures many free aromatic amines, it is not absolutely specific.

Since acid solutions of PASA decomposed rapidly upon heating over 50°, it was necessary in determining total amines to hydrolyze for eighteen hours at 38°. Values thus obtained with p-acetylaminosalicylic acid (AcPASA) were comparable to an equivalent PASA solution similarly treated or to at least 0.95 that of an unheated PASA solution. To effect hydrolysis 2.4 N HCl was used as it was found that 1.2 N HCl gave incomplete hydrolysis of AcPASA added to tissue and 6 N HCl delayed color development and gave higher blank readings.

The addition of PASA to minced tissues other than brain yielded 0.90 ± 0.05 recovery when the tissue was diluted 1:20 and precipitated with fifteen per cent trichloroacetic acid. Neither tungstic acid nor cadmium sulfate gave as good recovery. Recovery from brain was 0.80 ± 0.10 , from blood or plasma, 0.96 ± 0.03 . No difficulty was encountered in determining PASA directly from urine diluted 1:50 or more.

¹ Presented before the American Society for Pharmacology and Experimental Therapeutics, March, 1948. An abstract appears in *Federation Proceedings*, 7: 263, 1948.

Free phenol groups in urine were determined by a method essentially similar to that described by Brodie (14) for salicylates except that extraction with ethylene dichloride was omitted. Values thus obtained cannot be considered entirely reliable because it was found that the color intensity of AcPASA at 510 $m\mu$ was 1.35 that of PASA. No corrections in calculations were made because other conjugates of PASA were theoretically possible and their effects on color intensity were not known.

Procedure for free amine in urine. Reagents:

(1) Standard solutions of PASA in concentrations from 0.1 to 1.0 mgm. per cent can be made from a stock standard containing 100 mgm. PASA in one liter of distilled water. Solutions should be freshly prepared. Similarly, 128 mgm. AcPASA in one liter 0.1 N NaOH can be used as the stock solution to make working standards of lower concentrations.

(2) Hydrochloric acid approximately 2.4 N.

(3) Sodium nitrite—40 mgm. per cent.

(4) Ammonium sulfate—250 mgm. per cent in 50 per cent acetic acid.

(5) N-(1-naphthyl) ethylenediamine dihydrochloride—100 mgm. per cent.

(6) Trichloroacetic acid—15 per cent.

To 2 cc. of dilute urine add 2 cc. of 2.4 N HCl and chill in a mixture of ice and water. Add 1 cc. sodium nitrite reagent, shake vigorously and add 2 cc. of the ammonium sulfate solution. Shake vigorously for 10 seconds and immediately add 1 cc. of dye reagent. Allow at least 15 minutes for full development of color and read in a Coleman Junior Spectrophotometer at 540 $m\mu$, using a reagent blank (distilled water plus reagents) to set the instrument to zero optical density. Estimate the concentration of free amine from the standard curve.

Total amine in urine. To 2 cc. of diluted urine add 2 cc. of 2.4 N HCl, stopper with cotton and hydrolyze for eighteen hours at 38°, then proceed as for free amine.

Procedure for free and total amines in plasma. To 1 cc. plasma (or blood) add 15 cc. of distilled water (dilute more if necessary) plus 4 cc. of 15 per cent trichloroacetic acid. Shake vigorously, transfer to centrifuge tube and centrifuge for 5 minutes at 2500 RPM. Remove 2 cc. samples of clear supernatant layer (filter if cloudy) and proceed as directed for urine.

Procedure for rat tissue. Mince 1 part (one gram whenever possible) of tissue plus 9 or 10 parts of H₂O in a Waring Blendor. To 4 cc. of minced tissue, add 4 cc. of trichloroacetic acid and mix well. Transfer, centrifuge, then remove separate 2 cc. samples of clear supernatant layer (filter if necessary) for determination of free and total amine. Proceed as for urine.

Determination of PASA by free phenol in urine. Reagent-ferric nitrate, 1 per cent in 0.07 N HNO₃.

Add 10 cc. of dilute urine to 0.25 cc. of the iron reagent. Read in the Coleman Junior Spectrophotometer at 510 $m\mu$ with the instrument set to zero optical density with a reagent blank, and estimate the free phenol concentration from the standard curve.

ABSORPTION, DISTRIBUTION AND EXCRETION OF PASA IN RATS

Rats were given PASA, 200 mgm. per kgm. intravenously, and were then etherized immediately, fifteen minutes, two hours or four hours later. Various organs were removed and assayed for free and total amine in the manner previously described. The results as shown in table 1 indicate that by far the highest concentration of PASA was attained in the kidney, then lung and liver, with the latter yielding appreciable values for conjugated amine. These levels fell rapidly and within four hours, practically no PASA was detected in any tissue except the gastro-intestinal tract.

To ascertain whether the total administered dose of PASA could be accounted for, the drug concentration in the whole animal body was determined immedi-

TABLE 1

Concentration of *p*-aminosalicylic acid in rats after 200 mgm. per kgm. intravenously.

TIME SACRIFICED	MGm. PER KGm. FRESH TISSUE																	
	Blood		Kidney		Brain		Heart		Lung		Liver		Spleen		Muscle		GI tract	
	F	T	F	T	F	T	F	T	F	T	F	T	F	T	F	T	F	T
15 minutes	470	—	675	—	26	—	67	—	181	—	150	—	53	—	135	—	167	—
	260	280	602	642	25	48	105	112	128	156	127	167	74	84	100	104	109	139
	210	220	431	459	33	34	103	104	126	144	91	122	103	114	77	81	100	133
	410	400	837	837	27	24	147	147	211	228	169	188	95	99	115	120	123	140
2 hours	5	10	35	72	<5	9	13	25	7	22	11	17	5	16	10	15	—	—
	6	10	55	89	<5	9	11	13	6	14	11	18	7	16	10	10	44	121
	5	7	22	22	<5	<5	<5	<5	<5	6	<5	8	<5	<5	<5	<5	39	114
4 hours	<5	<5	10	16	10	9	<5	<5	<5	<5	9	11	<5	<5	<5	<5	39	122
	<5	<5	<5	7	<5	<5	<5	<5	<5	<5	6	6	<5	<5	—	—	52	111
	6	7	7	12	—	—	<5	<5	—	—	10	13	<5	<5	<5	<5	44	106
	<5	<5	<5	10	<5	<5	<5	<5	<5	<5	5	8	<5	6	<5	<5	163	200

F = free amine.

T = total amine.

TABLE 2

Distribution of *p*-aminosalicylic acid in rats after 200 mgm. per kgm. intravenously

TIME SACRIFICED	MGm. PER KGm. RAT CARCASS*			MGm. RECOVERED IN URINE				PER CENT OF TOTAL DOSE AC- COUNTED FOR
	Free NH ₂	Total NH ₂	Per cent recovered	Free NH ₂	Total NH ₂	Free OH	Per cent recovered	
Immediately	180†	200†	103	0	0	0	0	103
	217	222	93	0	0	0	0	93
	229	240	100	0	0	0	0	100
15 minutes	141	159	65	—	10	—	23	87
	117	117	49	12	14	14	30	79
	—	—	—	8	—	9	23	—
2 hours	29	39	16	19	20	20	58	76
	17	38	17	36	40	38	75	92
	11	17	7	41	47	48	98	105
	8	18	7	33	37	36	97	104
	24	39	16	—	—	—	—	—
	79†	99†	39	38	47	45	73	102
MGm. PASA PER KGm. GI TRACT								
2 hours	44	121	7	29	33	33	62	69
	38	111	6	23	30	31	83	89
4 hours	39	122	15	33	40	44	84	99
	52	111	5	76	27	29	74	79
	44	106	9	17	23	23	80	89
	163	200	14	37	42	40	93	107

* Animal minus skin and tail.

† Intravenous dose 160 mgm. per kgm.

‡ 25 per cent total dose administered intraperitoneally.

ately, fifteen minutes or two hours after intravenous administration of PASA. After the animals were stripped of their skins and tails, the carcass was minced thoroughly with its weight of water in a Waring blender, and further diluted with nine or ten parts of distilled water. A four cubic centimeter sample was treated as described in tissue determination. Total urine was collected and analyzed for free and total amine and free phenol. After two hours less than twenty per cent of the total dose administered was recovered from the animal carcass (table 2) and practically all the remainder in the urine.

To investigate further the extent of PASA storage in tissue, three rats were each given six single 200 mgm. per kgm. doses intraperitoneally over a forty-eight hour period. Twenty-four hours after the last injection, the concentration of free and total amine was determined in the animal carcass. The presence of PASA could not be demonstrated, indicating little if any storage of the compound.

TABLE 3

Distribution of p-aminosalicylic acid in rats sacrificed four hours after 200 mgm. per kgm. orally

MG/ PER KG/ CARCASS*			MG/ PER KG/ GI TRACT			MG/ IN URINE				PER CENT TOTAL DOSE ACCOUNTED FOR
F NH ₂	T NH ₂	Per cent T dose	F NH ₂	T NH ₂	Per cent T dose	F OH	F NH ₂	T NH ₂	Per cent T dose	
5	5	2	26	45	2	46	35	46	82	86
11	13	5	57	76	4	35	31	35	68	77
4	8	3	15	31	2	35	33	40	85	90
5	6	4	39	47	4	16	13	18	82	90

* Animal minus skin, tail and gastrointestinal tract.

F NH₂ = free amine

T NH₂ = total amine

T dose = total dose

The absorption of PASA was studied in a fourth group of rats by comparing the amount of PASA present in the total animal carcass (whole animal minus gastrointestinal tract, skin and tail), the entire gastrointestinal tract plus excreta and in the urine two and four hours after oral and intravenous administration. Rapid and total absorption of sodium PASA is indicated by the fact that within four hours approximately eighty per cent of the compound can be accounted for in the urine (table 3) and by the fact that gastrointestinal levels approximate those found after intravenous administration (table 2 and 3). Although the concentration of PASA in the gastrointestinal tract is rather low, it represents most of the PASA still remaining in the animal body. This suggests that the intestines are probably involved to a small extent in the excretion of p-aminosalicylic acid.

BINDING OF PASA BY PLASMA PROTEINS

Studies of the binding of PASA by plasma proteins were investigated on plasma obtained from individuals receiving the compound. The ultrafiltration

technique of Lavieties (15) was adopted, using Visking membranes. Experiments *in vitro* were performed with 50, 100 and 500 mgm. per liter of PASA dissolved in 3.5 percent bovine albumin buffered with 0.15 M phosphate to pH 7.4. No significant changes in PASA concentrations were noted in plasma allowed to stand during the period of ultrafiltration (usually overnight).

As can be ascertained from figure 1, at plasma levels from 40–100 mgm. per liter, approximately fifty to sixty percent is bound to plasma-proteins, presumably albumin. *In vitro* results with 3.5 percent crystalline bovine albumin containing 50, 100 and 500 mgm. per liter of PASA show respective bindings of 70, 60 and 50 percent.

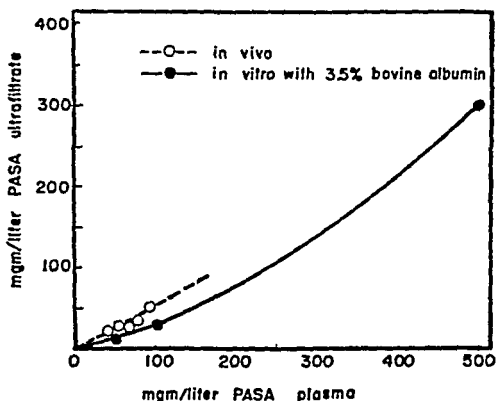


FIG. 1. BINDING OF PASA IN HUMAN PLASMA AND BOVINE ALBUMIN

DISTRIBUTION OF PASA IN DOG AND HUMAN BLOOD

In order to determine the distribution of PASA in blood, blood samples were taken from dogs and humans receiving a single dose of the compound (dogs intravenously; humans orally) using heparin as an anticoagulant. Free amine in whole blood and in plasma as well as apparent cell volume were determined and used to calculate the amount in red cells.

Data on the distribution of PASA in blood are summarized in table 4. The amount present in the red cells is relatively low. At fifteen minutes about seventy percent of the compound is present in the plasma and it remains unchanged at the end of three hours. In three individuals where the concentration of PASA in the plasma ultrafiltrate was simultaneously determined, the values obtained were of the same order of magnitude as the PASA concentration in cell water (table 5).

At the end of the fourth hour in many instances the calculated percent of free amine in the plasma appears to decrease. We hope to investigate these findings further on individuals who have received repeated doses of PASA.

TABLE 4
Distribution of p-aminosalicylic acid in dog and human blood

SUBJECT	TIME	BLOOD	PLASMA	ERYTHROCYTES*	PER CENT IN PLASMA
		mgm PASA/liter	mgm PASA/liter	mgm PASA/liter	
Dog A	hours				
	0:15	144	203	73.8	76
	1:00	92.5	127	52.2	74
	3:00	47.5	64.2	28.1	73
	5:00	24.5	24.5	24.6	54
Dog B	0:15	136	177	89.7	70
	3:00	26.3	43.2	7.2	87
	5:00	10.5	12.0	8.1	64
EL	1:00	36.6	57.3	16.2	82
	3:00	18.4	28.8	7.1	82
	5:00	16.5	16.6	16.4	56
JN	1:30	52.5	92.1	26.7	83
				32.0†	71
CD	1:30	—	21.0	9.3	70
MK	1:30	—	40.3	16.0	78
SK	1:00	61.0	82.0	37.3	74
	4:00	11.3	6.1	17.5	31
BG	1:00	57.2	99.0	22.8	79
	4:00	9.2	14.7	4.7	71
GC	1:15	78.0	123	29.4	89
JR	1:30	41.0	58.6	21.9	74
	4:00	9.2	15.8	20.4	89
AH	1:30	48.5	72.0	20.9	84
	4:00	12.6	6.3	19.8	28

* Concentration in cells $\frac{\text{whole blood conc.} - (\text{plasma volume} \times \text{plasma conc.})}{\text{cell volume}}$

† Determined values on packed cells

TABLE 5
Distribution and binding of p-aminosalicylic acid in human blood

	SUBJECTS		
	EL	MK	JN
	mgm PASA/liter	mgm PASA/liter	mgm PASA/liter
Whole blood	36.6	—	52.5
Plasma	57.3	40.3	92.1
Erythrocytes	16.2*	16.0	26.7* 32.0
Plasma ultrafiltrate	26.5	19.5	44.4
Cell water	22.5*	22.1*	44.5*
Per cent bound	61	52	48
Per cent in plasma	82	78	83

* Calculated values

PLASMA CURVE AND EXCRETION OF PASA IN DOGS

The plasma concentration of PASA was obtained on three dogs given 100 mgm. per kgm. of the sodium salt of p-aminosalicylic acid intravenously. After obtaining hematocrits, total blood, plasma and urine were determined for free and conjugated amines at various time intervals. Urine was also analyzed for free phenol.

The plasma-concentration time curve of PASA as obtained from averaging values determined on three dogs is shown in figure 2. The concentration of free amine in plasma falls from a level of approximately 200 mgm. per liter in fifteen minutes to less than 20 mgm. per liter within seven hours. On extrapolation of the log of plasma levels to zero, the values thus obtained and the dose in mgm.

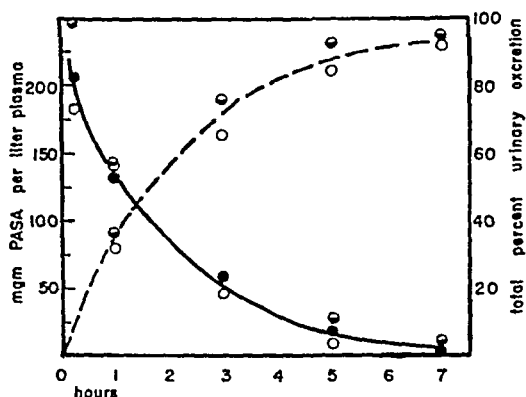


FIG. 2. CONCENTRATION OF PASA IN PLASMA AND ITS RATE OF EXCRETION IN THREE DOGS AFTER APPROXIMATELY 100 MGm. PER KGm. INTRAVENOUSLY

per kgm. for each dog were used to calculate the apparent volume of distribution in percent of body weight. The results obtained respectively in each dog were 33, 31 and 36 percent. If the assumption is made that dog plasma binds PASA to the same extent as human plasma these values would be roughly doubled, indicating penetration of the drug into at least some of the intracellular fluids.

Closely following the fall in plasma PASA is the rapid rate of urinary excretion of the compound. The percent of PASA excreted in the urine after the end of the first hour is somewhat higher than those shown in figure 2 as no rinsing of the bladder or catheter was carried out. Eighty to ninety percent of the injected PASA was accounted for as free amine or free phenol. This indicates that very little conjugation of the amine or phenol grouping of PASA occurs in the dogs after a single dose of the compound.

ABSORPTION AND EXCRETION OF PASA IN MAN

Blood levels and urinary excretion of PASA in man were made on student volunteers and on tubercular patients. Hematocrits, total blood, plasma and urine levels of PASA at various time intervals were obtained on student volun-

teers after single four gram oral doses of the sodium salt. Five tubercular patients received 2.5 grams at 6 hour intervals over a twenty-four or thirty hour period. PASA plasma levels were determined two to six hours after the last dose. Total collections were analyzed for free and conjugated amines and free phenol.

The plasma curve obtained from individuals taking a single oral 4 gram dose of the sodium salt of PASA is shown in figure 3. No correction was made for differences in body weight. Although there are individual variations, rapid and complete absorption of PASA is indicated by the fact that peak plasma levels occur between the first and second hour and by the fact that within seven hours

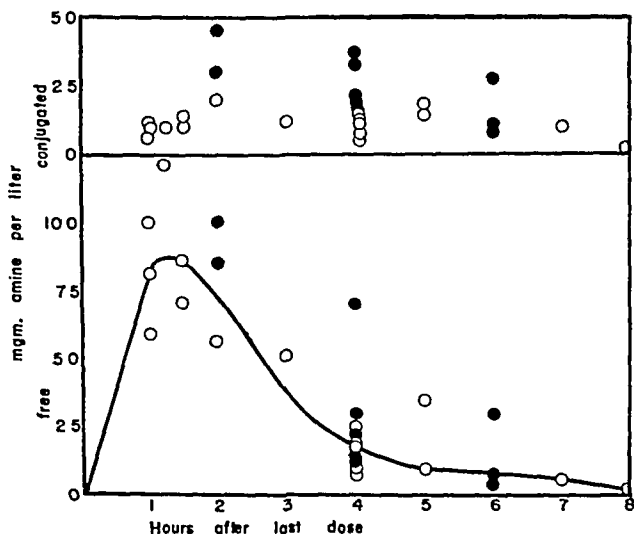


FIG. 3. HUMAN PLASMA CURVE OF PASA AFTER SINGLE 4 GRAM DOSE (OPEN CIRCLES AND REPEATED 2.5 GRAM DOSES (FILLED CIRCLES)

approximately eight-five percent of the compound can be accounted for in the urine (figure 4) as free and conjugated amines.

After repeated single oral 2.5 gram doses of PASA the plasma curve obtained for free amine after the last dose approximates that of the single 4 gram dose, while values for conjugated amine are slightly higher (figure 3). Eighty to eighty-five percent of the total amount given was accounted for in the urine collected during the period of administration and for the following ten hours (table 6). The continental workers who accounted for only thirty-five percent of the total dosage of PASA in urine (7) probably destroyed much of the PASA and AcPASA present by boiling with concentrated hydrochloric acid. The rapid excretion of PASA suggests that if it is necessary to maintain an active blood level for clinical effectiveness, it must be given at four hour intervals.

Recovery values of PASA as free phenol in urine are somewhat higher than for

PLASMA CURVE AND EXCRETION OF PASA IN DOGS

The plasma concentration of PASA was obtained on three dogs given 100 mgm. per kgm. of the sodium salt of p-aminosalicylic acid intravenously. After obtaining hematocrits, total blood, plasma and urine were determined for free and conjugated amines at various time intervals. Urine was also analyzed for free phenol.

The plasma-concentration time curve of PASA as obtained from averaging values determined on three dogs is shown in figure 2. The concentration of free amine in plasma falls from a level of approximately 200 mgm. per liter in fifteen minutes to less than 20 mgm. per liter within seven hours. On extrapolation of the log of plasma levels to zero, the values thus obtained and the dose in mgm.

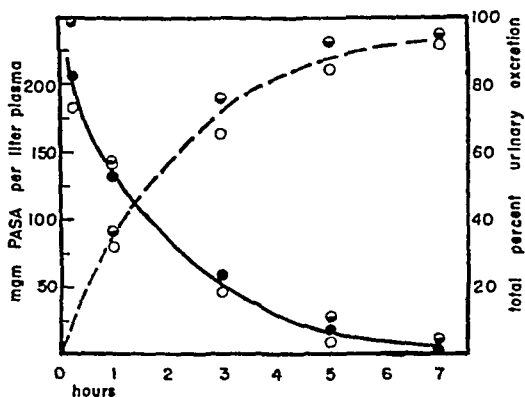


FIG. 2. CONCENTRATION OF PASA IN PLASMA AND ITS RATE OF EXCRETION IN THREE DOGS AFTER APPROXIMATELY 100 MGm. PER KGm. INTRAVENOUSLY

per kgm. for each dog were used to calculate the apparent volume of distribution in percent of body weight. The results obtained respectively in each dog were 33, 31 and 36 percent. If the assumption is made that dog plasma binds PASA to the same extent as human plasma these values would be roughly doubled, indicating penetration of the drug into at least some of the intracellular fluids.

Closely following the fall in plasma PASA is the rapid rate of urinary excretion of the compound. The percent of PASA excreted in the urine after the end of the first hour is somewhat higher than those shown in figure 2 as no rinsing of the bladder or catheter was carried out. Eighty to ninety percent of the injected PASA was accounted for as free amine or free phenol. This indicates that very little conjugation of the amine or phenol grouping of PASA occurs in the dogs after a single dose of the compound.

ABSORPTION AND EXCRETION OF PASA IN MAN

Blood levels and urinary excretion of PASA in man were made on student volunteers and on tubercular patients. Hematocrits, total blood, plasma and urine levels of PASA at various time intervals were obtained on student volun-

a long period of time, it would seem wise to administer the compound only as the sodium salt or in conjunction with adequate amounts of sodium bicarbonate. An additional advantage may be that the continued administration of high doses of the sodium salt will not affect the alkali reserve of the body, whereas, with continued treatment with the free acid it is highly possible for an acidosis to develop. Furthermore, it is our experience that the sodium salt appears to cause less nausea in patients.

ULTRA-VIOLET SPECTROPHOTOMETRIC STUDIES ON PASA

Spectrophotometric measurements were carried out in a Beckman Model DU photoelectric quartz spectrophotometer. As can be ascertained from

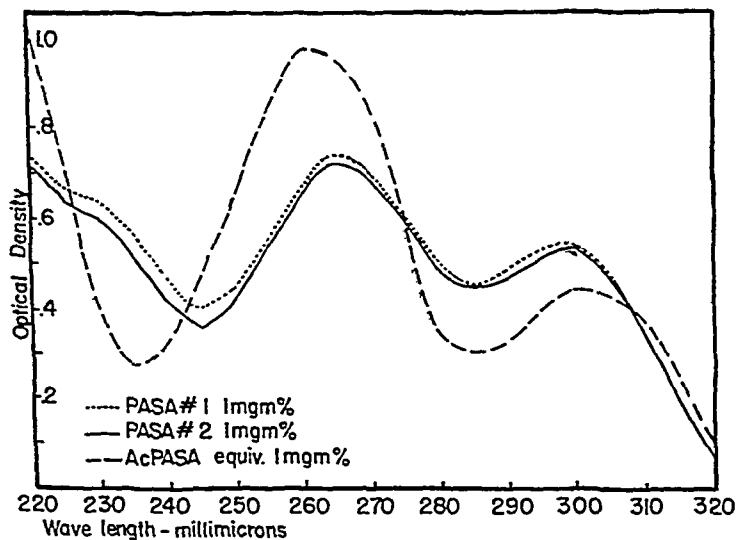


FIG. 5. ULTRAVIOLET ABSORPTION CURVES OF PASA AND AcPASA

figure 5, the ultra-violet absorption spectrum of PASA indicates a peak at 266 $m\mu$ and another at 298 $m\mu$. Two samples of PASA (10 mgm. per liter) obtained from different commercial houses gave essentially the same curve, although the lighter colored preparation gave slightly higher readings. An equivalent solution of AcPASA shows maxima at 261 $m\mu$ and 301 $m\mu$, approximately the same region for PASA. The first peak of AcPASA, however, is somewhat higher and broader than that for PASA. Consequently, when it was found that man conjugates PASA to an appreciable degree, extensive spectrophotometric studies were not made. However, an ether extract of a urine sample buffered in pH 3.2 acetate from a dog given PASA exhibited a spectrum which appeared to closely coincide with that of an ether extract of PASA (figure 6).

free plus conjugated amines but if correction is made for the higher color intensity given by conjugated amines with iron, the values closely approximate those for total amine. However, this correction is not entirely valid for reasons previously stated under methods.

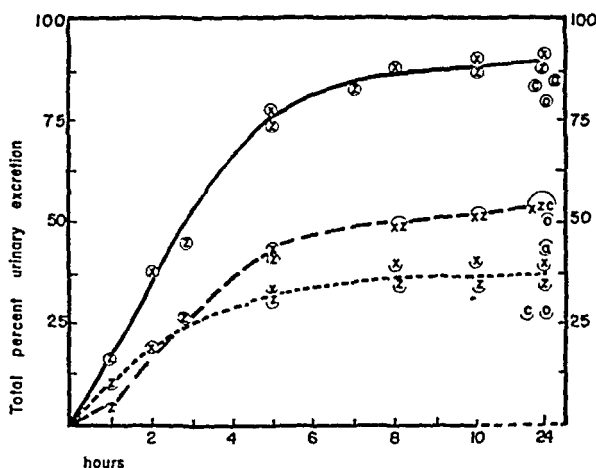


FIG. 4. RATE OF EXCRETION AND PER CENT RECOVERY OF PASA IN INDIVIDUALS AFTER A SINGLE ORAL 4 GRAM DOSE

Straight line denotes total amines, dashed line conjugated amine and dotted line free amine

TABLE 6

Per cent of PASA recovered in urine of individuals receiving repeated single 2.5 gram doses every 4-6 hours

Total urine was collected throughout the experiment and for the following ten hours

PATIENT	NO. OF DOSES	TOTAL GRAMS	F-NH ₂	C-NH ₂	T-NH ₂	F-OH
DH♂	4	10	3.2	49	81	94
TB♂	6	15	21	64	85	106
*TW♂	6	15	27	42	69	77
FG♀	5	12.5	45	39	84	93

* Total urine collected until 6 hours after last dose.

F = free, C = conjugated, T = total, NH₂ = amine, OH = phenol.

In contrast to the findings on the rat and the dog, humans conjugate PASA to a much greater extent. As can be ascertained from table 6, roughly fifty to seventy-five percent of the amines in the urine of humans is in the conjugated form. This finding may be of practical importance because we have found that p-acetylamino salicylic acid is quite insoluble in acid but its solubility increases considerably with an increase in pH. To minimize the dangers of possible renal damage in tubercular patients who would be receiving high doses of PASA over

distribution of the urinary amines in an acetate pH 3.2-ether, -ethylene dichloride or -isoamyl alcohol system was compared with that of PASA and AcPASA respectively. A 3 cc. sample of diluted urine was equilibrated with 3 cc. of acetate buffer and 20 cc. of each solvent by vigorous shaking. The solvent was then removed and the aqueous layer was analyzed for unextracted amines.

The results are summarized in table 7. It was found that the urinary amines were extracted to a lesser extent than the standards, thus indicating the presence of one or more amines which are more soluble in water. Considerable species difference is also apparent. As indicated in the results, man converts PASA to more water soluble derivatives to a greater extent than either the rat or dog.

Some qualitative tests were made on the urine of various individuals given PASA in order to ascertain something of the nature of the water soluble metabolites. Only slight or no reducing properties were obtained with Benedict's solution. Tests for glycuronate using Dische's method (20) also yielded negative or only slightly positive results. These findings are in contrast to results obtained with two closely related derivatives of PASA, namely p-aminobenzoic and salicylic acid, both of which give rise to a considerable increase in urinary glycuronates (11, 19).

COUNTERCURRENT STUDIES ON URINE METABOLITES OF PASA

Fractionation and characterization of urinary products was carried out using a modification of Craig's countercurrent technique (16, 17).

Procedure. Total twenty-four hour urine was collected from an individual who received ten grams of the sodium salt of PASA in divided doses over a twelve hour period and analyzed for free and conjugated amines. A 10 cc. sample of urine was then diluted with 40 cc. of 2 M acetate buffer pH 3.35 (proportions of a mixture previously determined to give a final pH of 3.40) and a sixteen transfer separation was effected in a system consisting of equal parts of isoamyl alcohol and 1.6M acetate buffer pH 3.40. The lower aqueous acetate phase was allowed to migrate and its transfer from one bottle to another was facilitated by means of a long hypodermic needle attached to a 50 cc. syringe. Some difficulty was encountered in carrying out the process. Emulsion formation occasionally caused delays and made separation of the two layers difficult. At the end of the distribution procedure, bottle 0 was found to contain only about 40 cc. of the isoamyl layer.

The lower acetate layers were then determined for free and conjugated amines by the usual method. The amines in the isoamyl alcohol were determined by extracting 1 cc. of the upper layer with 10 cc. of 0.1 N or 0.5 N NaOH and then analyzing the aqueous alkaline layer in the usual manner. The values were then corrected for the amount lost in the extraction. The total amounts of free and conjugated amines in each bottle were thus determined. The characteristic distribution curves were then obtained by plotting the fraction of the total amount of free and conjugated amines present in each bottle against the number of the bottle. The experimental curves were then compared with the theoretical ones calculated in the manner described by Williamson and Craig (18).

The distribution pattern of free and conjugated amines obtained on the urine of one individual is shown in figure 7. It is quite obvious that sixteen transfers did not effect complete separation of the constituents, but sufficient fractionation occurred to indicate that at least three free and two conjugated amines were present. If an attempt is made to calculate a theoretical curve to get the experimental free amine distribution a reasonable approximation is obtained if

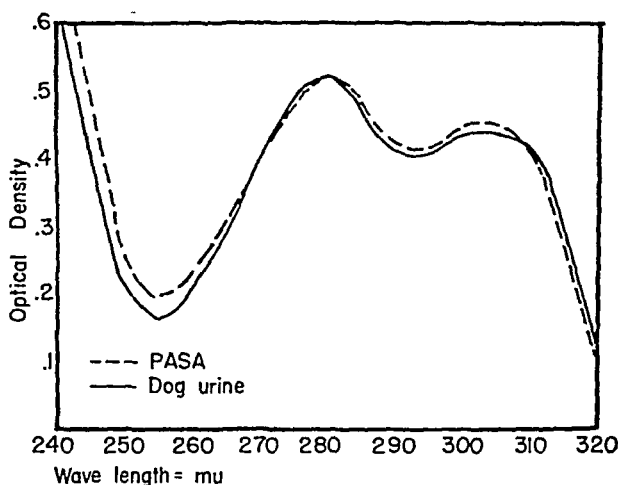


FIG. 6. ULTRAVIOLET ABSORPTION CURVES OF ETHER EXTRACTS OF PASA AND URINE OF DOG GIVEN PASA

TABLE 7

Distribution of PASA, AcPASA and urine metabolites of the rat, dog and man buffered with 2 M acetate pH 3.2 after equilibrating with various solvents

	PER CENT UNEXTRACTED					
	ETHER		ETHYLENE DICHLORIDE		ISOAMYL ALCOHOL	
	F	C	F	C	F	C
Standard	1	1	41	81	3	4
Rats { Group I (3) Group II (3)	10	0?	46	103?	15	0?
	16	—	51	—	17	—
Dogs { A B	17	—	51	—	—	—
	25	—	52	—	—	—
Humans { DH TW FG TB.	40	23	66	85	26	14
	39	39	64	93	—	—
	43	38	74	85	—	—
	39	41	69	83	—	—

F = free amine.

C = conjugated amine.

? = values too low for accurate determination.

PARTITION COEFFICIENTS OF PASA, AcPASA AND URINARY PRODUCTS

As a preliminary before application of the countercurrent technique, the solubility characteristics of the urinary amines found in the rat, dog and man given PASA were first investigated by a single extraction procedure. The

eighteen percent is PASA, thirteen percent is p-aminosalicyluric acid and remainder represents one free amine and one conjugated amine which are highly water soluble. None of the fractions gave a positive test for glycuronates. Further studies are necessary to establish the identity of these metabolites.

ACKNOWLEDGEMENTS. We are grateful to the Office of Naval Research for generously supporting our studies, to Dr. K. G. Rosdahl of A. B. Ferrosan, Malmö, Sweden and Dr. A. C. Bratton of Parke-Davis and Co. for supplying p-aminosalicylic acid, to Dr. H. B. Lewis for the p-aminosalicyluric acid, to Dr. Celia White Tabor for running the glycuronate tests, to Dr. R. J. Madden for synthesizing acetylated p-aminosalicylic acid, to Drs. Sol Katz, Bernard Miller and James Feffer for their cooperation with the tubercular patients, to Drs. E. W. Ligon, Jr., and C. D. Leake for their comments, to Miss Anne-Lise Schlytter for translating the Scandinavian publications, to Miss Helen Ross for her assistance, and to all the student volunteers for their enthusiastic cooperation.

SUMMARY

(1) Methods are presented for the rapid determination of p-aminosalicylic acid (PASA) in urine, plasma or tissues as free amine, conjugated amines, or free phenol.

(2) In various studies on rats, dogs and humans the sodium salt of PASA was found to be rapidly and totally absorbed and rapidly excreted. About eighty-five percent of the administered dose can be accounted for in the urine within ten hours.

(3) After administration of PASA, recovery of one or more conjugated amines is highest in man (sixty per cent of total dose), next in the rat (ten per cent) and least in the dog (zero per cent).

(4) Three compounds containing a free amino group and two compounds containing a conjugated amino group were separated in the urine of one individual taking PASA. Two of the free amino compounds are believed to be unchanged PASA and p-aminosalicyluric acid and one of the conjugated amino compounds to be acetylated PASA.

(5) No demonstrable storage of PASA occurs after single or repeated doses in rats. By far the highest concentrations are temporarily attained in the kidney, the lung and liver, and appreciable amounts are bound by plasma proteins.

(6) After a single oral four gram dose or following repeated two and one-half gram doses every six hour in humans, a maximum level of approximately 100 mgm. per liter is rapidly attained which falls to less than 10 mgm. per liter within six hours.

(7) It is recommended that PASA be administered clinically as the sodium salt and/or with adequate amounts of sodium bicarbonate to guard against crystalluria, acidosis and to decrease nausea.

REFERENCES

1. LEHMAN, J.: *Lancet*, 250: 15, 1946
2. LEHMAN, J.: *Svenska lak. tidning*, 43: 2029, 1946.
3. SIEVERS, O.: *Svenska lak. tidning*, 43: 2041, 1946.

three amines with respective partition coefficients 6.10, 0.76 and 0.054 are present in the hypothetical proportions of 52, 37, 11 percent. The theoretical points to be expected from summation of the three curves are indicated in the chart. Likewise the theoretical points for conjugated amines were calculated on the assumption that 90 percent of a compound with a partition coefficient of 5.95 and 10 percent of another with a partition coefficient of 0.17 were present. The low experimental values in bottle O were probably due to the experimental loss of part of the upper isoamyl layer to the other bottles.

In order to further characterize some of the urinary fractions, a comparison was made of the partition coefficients of the material from each maximum point of the experimental distribution curve with those obtained of compounds we had

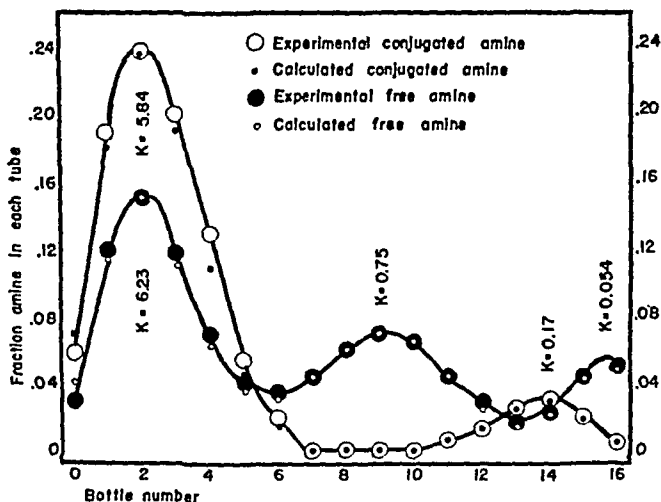


FIG. 7. DISTRIBUTION CURVE OF FREE AND CONJUGATED AMINES IN URINE OF ONE INDIVIDUAL AFTER TAKING 10 GRAMS OF PASA IN FOUR DIVIDED DOSES

on hand. In bottle two, the partition coefficients of the free and the conjugated amine were found to be 6.23 and 5.84 respectively. When the partition coefficients of PASA and AcPASA were determined for the same system as used in the counter-current distribution, the values obtained respectively were 6.18 and 5.90. There is reasonable evidence, therefore, to suggest that bottle two contains PASA and AcPASA.

At another maximum point of the distribution curve, namely bottle nine, the partition coefficient of the free amine present was found to be 0.75. The partition coefficient of p-aminosalicylic acid (2-hydroxy-4-amino-hippuric acid) for the same system was determined to be 0.74, indicating, therefore, that the substance in bottle nine is probably p-aminosalicylic acid.

If such is assumed to be the case, then of the total urinary amines excreted by this individual after taking PASA, approximately fifty-nine percent is AcPASA,

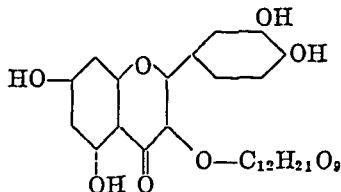
EFFECT OF RUTIN ON COAGULATION TIME OF RAT BLOOD

M. B. PLUNGIAN, JAMES C. MUNCH, AND J. B. WOLFFE

Department of Research, School of Pharmacy, Temple University, Philadelphia, Pa. and Wolfe Clinic, Philadelphia, Pa.

Received for publication September 4, 1947

Rutin is a flavonol-glycoside which undergoes hydrolysis with acid to yield quercetin, dextrose and rhamnose. Its structural formula was established by Attree and Perkin (1) in 1927.



In 1936 Szent-Gyorgyi and associates (2) prepared an extract from lemon peel named Hesperidin (another flavonol sugar chemically related to rutin), which reduced capillary fragility and decreased capillary permeability. Rutin was found in a variety of plants, including tobacco, garden rue, forsythia, elder flowers, violets and buckwheat (3, 4, 5). Couch and associates obtained a yield of 0.4 per cent of rutin from tobacco leaf as compared with 2.98 per cent from buckwheat plants three weeks old, and buckwheat now serves as the principle source of supply.

Clinical studies by Griffith and associates (6) were made on 1600 white-skinned hypertensives. Of this group increased capillary fragility appeared in 306, and 198 persons were followed in detail for an average of eleven months. Hemorrhagic complications such as apoplexy and retinal hemorrhages were more common in the group showing increased fragility. Administration of rutin usually reduced increased capillary fragility to normal and appeared to protect against such hemorrhagic complications. Ambrose and DeEds (7) have reviewed literature and shown that rutin decreased cutaneous capillary permeability as determined by the method employed. Further clinical studies by Shanno (8) showed that the administration of rutin was effective in decreasing pulmonary bleeding in two cases which had resisted other treatment. Kushlan (9) successfully checked intestinal bleeding in a patient with hereditary hemorrhagic telangiectasia by the administration of rutin. This led us to wonder what effect rutin might exert on the coagulation of blood, a point not found in available literature. This investigation was conducted to learn the effect of administration of rutin alone, as compared with combinations or mixtures of rutin with dicumarol or with bile salts, on the coagulation time of the blood of rats, as related to checking of hemorrhage (6, 8).

4. VALENTIN, G.: Svenska lak. tidning, 43: 2047, 1946.
5. YOUMANS, G. P.: Quart. Bull. Northwest. U. Med. Sch., 20: 420, 1946.
6. FELDMAN, W. H., KARLSON, A. G., AND HINSHAW, H. C.: Proc. Staff Meetings Mayo Clin., 22: 473, 1947.
7. ALIN, K., AND DIFS, H.: Nordisk Med., 33: 151, 1947. (Translation by E. I. Hoegberg issued by American Cyanamid Co.)
8. YOUMANS, G. P., RALEIGH, G. W., AND YOUMANS, A. S.: J. Bact. 54: 409, 1947.
9. SMITH, P. K., BAYLISS, J. R., ORGORZALEK, S., AND McCLURE, M. M.: Fed. Proc., 5: 154, 1946.
10. TABOR, C. W., BAILY, J., AND SMITH, P. K.: Fed. Proc., 6: 376, 1947.
11. TABOR, C. W., BAILY, J., AND SMITH, P. K.: Fed. Proc., 7: 258, 1948.
12. SMITH, P. K., GLEASON, H. L., STOLL, C. G., AND ORGORZALEK, S.: This Journal, 87: 237, 1946.
13. BRATTON, A. C., AND MARSHALL, E. K., JR.: J. Biol. Chem., 128: 537, 1939.
14. BRODIE, B. B., UDENFRIEND, S., AND COBURN, A. F.: This Journal, 80: 114, 1944.
15. LAVIETIES, P. H.: J. Biol. Chem., 120: 267, 1937.
16. CRAIG, L. C.: J. Biol. Chem., 155: 519, 1944.
17. CRAIG, L. C., COLUMBIE, C., MIGHTON, H., AND TITUS, E.: J. Biol. Chem., 161: 321, 1945.
18. WILLIAMSON, B., AND CRAIG, L. C.: J. Biol. Chem., 168: 687, 1947.
19. KAPP, E. M., AND COBURN, A. F.: J. Biol. Chem., 145: 549, 1942.
20. DISCHE, Z.: J. Biol. Chem., 167: 189, 1947.
21. VENKATARAMAN, A., VENKATARAMAN, P. R., AND LEWIS, H. B.: J. Biol. Chem., 173: 641, 1948.
22. McCLOSKEY, W. T., SMITH, M. I., AND FRIAS, J. E. G.: This Journal, 92: 447, 1948.

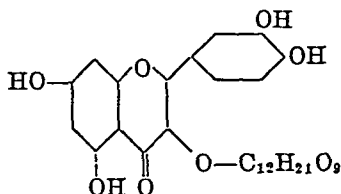
EFFECT OF RUTIN ON COAGULATION TIME OF RAT BLOOD

M. B. PLUNGIAN, JAMES C. MUNCH, AND J. B. WOLFFE

Department of Research, School of Pharmacy, Temple University, Philadelphia, Pa. and Wolfe Clinic, Philadelphia, Pa.

Received for publication September 4, 1947

Rutin is a flavonol-glycoside which undergoes hydrolysis with acid to yield quercetin, dextrose and rhamnose. Its structural formula was established by Attree and Perkin (1) in 1927.



In 1936 Szent-Gyorgyi and associates (2) prepared an extract from lemon peel named Hesperidin (another flavonol sugar chemically related to rutin), which reduced capillary fragility and decreased capillary permeability. Rutin was found in a variety of plants, including tobacco, garden rue, forsythia, elder flowers, violets and buckwheat (3, 4, 5). Couch and associates obtained a yield of 0.4 per cent of rutin from tobacco leaf as compared with 2.98 per cent from buckwheat plants three weeks old, and buckwheat now serves as the principle source of supply.

Clinical studies by Griffith and associates (6) were made on 1600 white-skinned hypertensives. Of this group increased capillary fragility appeared in 306, and 198 persons were followed in detail for an average of eleven months. Hemorrhagic complications such as apoplexy and retinal hemorrhages were more common in the group showing increased fragility. Administration of rutin usually reduced increased capillary fragility to normal and appeared to protect against such hemorrhagic complications. Ambrose and DeEds (7) have reviewed literature and shown that rutin decreased cutaneous capillary permeability as determined by the method employed. Further clinical studies by Shanno (8) showed that the administration of rutin was effective in decreasing pulmonary bleeding in two cases which had resisted other treatment. Kushlan (9) successfully checked intestinal bleeding in a patient with hereditary hemorrhagic telangiectasia by the administration of rutin. This led us to wonder what effect rutin might exert on the coagulation of blood, a point not found in available literature. This investigation was conducted to learn the effect of administration of rutin alone, as compared with combinations or mixtures of rutin with dicumarol or with bile salts, on the coagulation time of the blood of rats, as related to checking of hemorrhage (6, 8).

All doses were administered orally to Wistar strain albino rats, having an average weight of 200 grams. In a preliminary study tests were made to determine the threshold doses for rats which would affect coagulation time. It was found that the threshold effective dose of rutin alone was 14 mg., and of dicumarol alone was 8 mg. for rats weighing 200 to 250 grams. In this investigation litter mate female rats were used. They were fed on a diet of Purina Dog Chow, and had water *ad libitum*. Weighed doses of each drug or combination of drugs were suspended in 1 cc. of water, the suspension drawn into a Luer syringe with blunted needle and introduced into the stomach to insure accuracy of dosage. All doses were administered on the basis of mg. of drugs per rat, not mg./kg. body weight, although all rats were weighed before starting the investigation. Doses were given six days weekly for a period of six weeks, with the exception of the group receiving bile salts, which were under treatment for only three weeks. In general eight rats were used in each group under test, except the control and the bile salts group which consisted of three rats each.

The rutin used in this investigation was supplied by Dr. James F. Couch. The dicumarol was purchased on the open market. The bile salts were prepared by extracting sodium glycocholate and sodium taurocholate in the commercial lot of dried ox bile, and mixing these products in equal proportions for use.

The coagulation time of rat blood was determined by the capillary tube method developed by Sabraze (10) for human blood. A sample of blood was drawn into a glass tube with an inside diameter of 0.8 mm. until three-fourths of the tube was filled. The tube was rotated up and down to observe the flow of blood until no flow was noted. The interval between placing blood in the tube and time when coagulation developed was read with a stop watch. The tube was then broken to confirm the coagulation as evidenced by the appearance of a thread connecting the broken segments.

The vein at the end of the tail was nicked and samples of blood taken from each rat before starting treatment, and at intervals of three hours and twenty-four hours after the administration of a dose of the drugs used. The control group was given no medication, but bled at the same time as the experimental rats. In addition samples of blood were taken forty-eight hours after the last medication over the week-end; that is doses of drugs were administered on Saturday and samples drawn on Sunday and again on Monday morning immediately before administration of the next dose.

Blood from unmedicated rats showed coagulation time of approximately 90 seconds, when measured by this method. When successive samples of blood were taken from the same rat, coagulation time agreed within 5 seconds, or less. In studies on forty-eight rats the range of coagulation time before medication was started was from 70 seconds to 100 seconds, with an average value of 89.2 seconds and a standard deviation of 0.85 seconds for this mean value.

The average coagulation time for rats in a group in the control and in the groups treated with each of the drugs are shown in Table I. For the group in the control period, the average coagulation time was 89 seconds before drugs were given to any rats in this group. Samples of blood taken 3 hours, 24 hours and 48 hours after the period corresponding to administration of drugs in the treated groups of rats showed decreases of 2 per cent, 3 per cent and 1 per cent in the rate of coagulation, as compared with the original values of 89 seconds. Similarly results at the end of weekly intervals, including the sixth week, indicate that there was no significant change in the rate of coagulation of the blood of rats in this control group.

The average coagulation time for the group to receive rutin was 90 seconds; after treatment there was a progressive decrease in the coagulation time, and essentially equivalent values were noted for the 3 hour, 24 hour and 48 hour

samples. The average time for the group to receive bile salts was 87 seconds; during treatment there was a decrease in coagulation time, which was less than that produced in the rutin group. This decrease following bile salts is in agreement with the findings of Butt and co-workers (11) and of Warner et al (12) that

TABLE I

Percentage changes in coagulation time of rat blood following treatment

	WEEKS OF TREATMENT																	
	1 week			2 weeks			3 weeks			4 weeks			5 weeks			6 weeks		
	Hours after admin.																	
	3	24	48	3	24	48	3	24	48	3	24	48	3	24	48	3	24	
Control (89 sec.)..	-2	-3	-1	-3	-3	-7	-1	3	-2	-3	-3	1	-2	-3	-2	-2	-2	
Rutin (90 sec.)..	-26	-26	-27	-39	-40	-39	-56	-52	-56	-59	-61	-59	-63	-63	-66	-64	-67	
Bile Salts (87 sec.)..	-20	-14	-17	-17	-18	-27	-13	-23	—	—	—	—	—	—	—	—	—	
Dicumarol (91 sec.)..	64	67	31	82	64	41	70	63	85	59	63	28	62	37	33	75	46	
Rutin & Dicu- marol																		
Calc....	38	41	4	43	24	2	14	11	29	0	2	-31	-1	-26	-33	12	-21	
Obs.....	22	19	18	1	1	-19	-6	-12	-34	-35	-33	-45	-36	-40	-44	-37	-43	
Rutin & Bile Salts																		
Calc....	-46	-40	-44	-56	-58	-66	-69	-75	—	—	—	—	—	—	—	—	—	
Obs.....	-54	-56	-64	-67	-70	-72	-67	-67	-74	-74	-72	-73	-76	-74	-76	-70	-73	
Rutin & Bile Salts & Di- cuma- rol																		
Calc....	18	-27	-13	26	6	-25	1	-12	—	—	—	—	—	—	—	—	—	
Obs.....	-8	-10	-18	-19	-20	-19	-23	-25	-31	-21	-20	-19	-29	-36	-26	-33	-30	

simultaneous administration of bile salts and Vitamin K were effective in the treatment of hemorrhagic diathesis in human jaundice.

On the other hand the group receiving dicumarol showed a definite increase in coagulation time, as expected. The greatest increase was noted in the 3 hour samples, the 24 hour samples being usually less marked in their changes. The 48 hour samples showed much smaller changes, indicating that the dicumarol effect was transitory, as contrasted with the effect produced by rutin.

These doses of rutin and dicumarol together were administered to another group of rats. The coagulation shortening time of rutin was overcome by the prolonging action of dicumarol. The observed times of coagulation were less than the calculated times, based on the behaviors of the drugs administered alone, suggesting that the rutin effect tended to overcome that of dicumarol. This effect changed with time in the same way that the dicumarol effect had altered.

Administration of combinations of rutin and bile salts produced additive or potentiating prolongation in coagulation time. The observed coagulation times were longer than had been estimated: additional studies will be needed to indicate whether this is due to variations in individual animals, or to a true potentiation of effect.

Finally combinations of the three products (rutin, bile salts and dicumarol) were given to another group of rats. The observed effects were generally indicative of shorter coagulation time than expected; the rutin and bile salts appeared to produce a greater effect in this proportion than did the dicumarol. We have no information to show the effect of different proportions of these products.

These observations of the changes in coagulation time of the blood of albino rats after the administration of rutin, bile salts and dicumarol are being extended to other species of animals and also to man.

CONCLUSIONS

1. The coagulation time of the blood of Wistar strain albino rats was not significantly altered by collecting blood from the tail vein twice daily over a period of six weeks.

2. Oral administration of 14 mg. of rutin, or 30 mg. of bile salts decreased coagulation time of the rat's blood. Administration of 6 mg. of dicumarol increased the coagulation time.

3. When administered in combination, 14 mg. of rutin and 8 mg. of dicumarol tended to antagonize the effects of the drugs given alone, the net result being a decrease in coagulation time.

4. Administration of rutin and bile salts showed an additive decrease in coagulation time.

5. Administration of these doses of rutin, dicumarol and bile salts showed a decrease in coagulation time.

6. The same types of decreases were generally observed in samples of blood drawn 3 hours, 24 hours and 48 hours after the administration of the product.

REFERENCES

1. ATTREE, C. F. AND PERKIN, A. G.: *J. Chem. Soc.* 234, 1927.
2. ARMENTANO, P. L., BONTATH, A., BERES, T., RUSZNYAK, I., AND SZENT-GYORGYI, A.: *Deut. med. Wochschr.* 62: 1326, 1936.
3. COUCH, J. F., AND KREWSON, C. F.: United States Department of Agriculture Mimeograph Circular AIC-52, 1944.
4. COUCH, J. F., KREWSON, C. F., NAGHSKI, J., AND CORLEY, N. J.: United States Department of Agriculture Mimeograph Circular AIC-115, 1946.
5. COUCH, J. F., NAGHSKI, J., AND KREWSON, C. F.: *Science*, 103, 197, 1946.

6. GRIFFITH, J. Q., LINDAUER, M. A., SHANNO, R. L., AND COUCH, J. F. Scientific Exhibit. Am. Med. Assoc., San Francisco, July 1-5, 1946. Mimeographed.
7. AMBROSE, A. M., AND DEEDS, F.: THIS JOURNAL, 90:359, 1947.
8. SHANNO, R. L.: Am. J. Med. Sci. 211: 539, 1946.
9. KUSHLAN, S. D.: Gastroenterology 7: 199, 1946.
10. SABRAZE, through KOLMER AND BOERNER, Approved Laboratory Technic, p. 99, 1945.
11. BUTT, H. R., SNELL, A. M., AND OSTERBERG, A. E.: Proc. Staff Mayo Clinic 13: 74, 1938.
12. WARNER, E. D., BRINKHOUS, K. M., AND SMITH, H. P.: Proc. Soc. Exp. Biol. Med. 37: 628, 1938.

A COMPARATIVE STUDY OF THE ACTIVITY AND TOXICITY OF HEXYLCAINE (1-CYCLOHEXYLAMINO-2-PROPYLBENZOATE); A NEW LOCAL ANESTHETIC AGENT

KARL H. BEYER, ALBERT R. LATVEN, WALTER A. FREYBURGER,
AND MARY P. PARKER

*Department of Pharmacology, The Medical Research Division, Sharp and Dohme, Inc.,
Glenolden, Pennsylvania*

Received for publication December 30, 1947

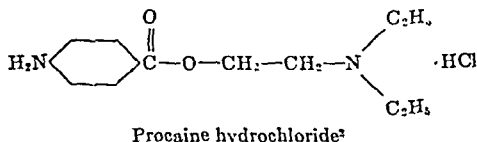
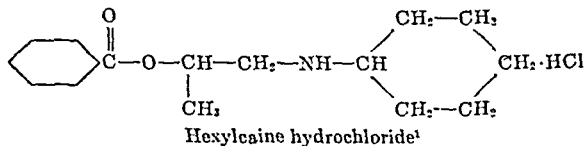
It is often stated that among useful local anesthetic agents inherent activity and toxicity go more or less hand-in-hand. Fortunately, a systematic evaluation of a series of related agents will show that the above generalization is by no means invariable. Thus there is justification for a continued desire for better and more generally useful local anesthetic agents. It follows that the goal in undertaking a research program in anesthetic agents is the greatest attainable exception to the generalization stated above; i.e., a compound that combines a greater spectrum of useful local anesthetic activity with a greater margin of safety. Cope and his associates synthesized over a hundred agents for this purpose (1). The preliminary evaluation of a large number of these compounds has been undertaken by Kuna and Seeler (2).

We have submitted fifteen of the compounds synthesized by Cope to an extensive pharmacologic evaluation. From among these agents a new compound, hexylcaine,¹ manifested the desirable attributes of a generally useful, reasonably safe, local anesthetic agent. It was considered desirable to evaluate certain other well established local anesthetic drugs under these same conditions. This has been done throughout the studies.

The purpose of this report is to present the relative activity and toxicity of hexylcaine and six widely used local anesthetic agents.

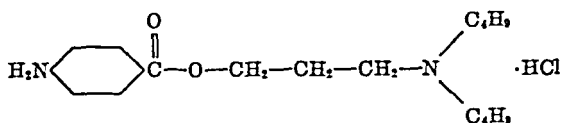
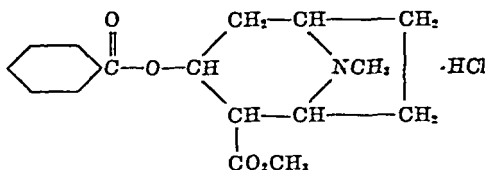
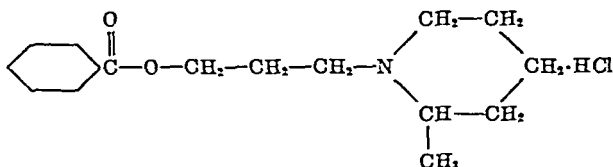
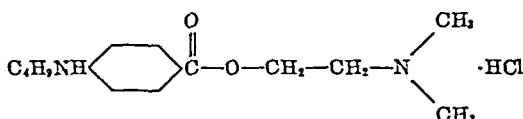
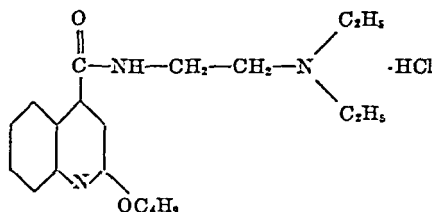
CHEMISTRY

The structural formulae for the compounds used in this study are presented herein for comparison and convenience in future references.



¹ Hexylcaine is the non-proprietary name for 1-cyclohexylamino-2-propylbenzoate.
Cyclaine is the Sharp and Dohme, Inc. trademark for this compound.

² Procaine is the p-aminobenzoic acid ester of diethylaminoethanol.

Butacaine hydrochloride¹Cocaine hydrochloride⁴Neohesin hydrochloride⁵Tetracaine hydrochloride⁶Dibucaine hydrochloride⁷

¹ Butacaine is the non-proprietary name for the p-aminobenzoic acid ester of γ -N-dibutylaminopropanol. Butyn is the Abbott Laboratories, Inc. trademark for this compound.

⁴ Cocaine is benzoylmethylecgonine.

⁵ Neohesin is a non-proprietary name for benzoyl- γ -(2-methylpiperidino)-propanol. Metycaine is the Eli Lilly and Company trademark for this compound.

⁶ Tetracaine is the non-proprietary name for the p-N-butyl-aminobenzoic acid ester of β -N-dimethylaminoethanol. Pontocaine is the Winthrop Chemical Co., Inc. trademark for this compound.

⁷ Dibucaine is the non-proprietary name for the β -diethylaminoethylamide of 2-butoxy-cinchoninic acid. Nupercaine is the Ciba Pharmaceutical Products, Inc. trademark for this compound.

Hexylcaine is 1-cyclohexylamino-2-propylbenzoate. Its synthesis has been accomplished by Cope and Hancock (1). The hydrochloride of hexylcaine is soluble in water to the extent of about 12 per cent. A one per cent solution of the hydrochloride is stable to boiling and autoclaving for sterilization purposes. The molecular weight of the base is 261.35. The physical and chemical characteristics of the other compounds listed above are described in New and Non-official Remedies (3) or the U. S. Pharmacopoeia XIII (4).

TOXICITY STUDIES

The intravenous LD_{50} of local anesthetic agents is a reflection of their inherent toxicity, when administered rapidly to several species of animals. Thus one can

TABLE 1

The intravenous toxicity of a series of local anesthetic agents to mice, guinea pigs, and rabbits

The results are expressed as LD_{50} , in terms of mg. of base/kg. of body weight.

AGENT	LD_{50} , MG. (BASE)/KG. BODY WT.					
	Mice	No. of animals	Guinea pigs	No. of animals	Rabbits	No. of animals
Procaine	78 \pm 5	60	56 \pm 4	25	41 \pm 2	25
Neethesin	32 \pm 4	40	16 \pm 2	30	18 \pm 6	35
Hexylcaine	24 \pm 2	60	17 \pm 2	25	14 \pm 2	20
Cocaine	25 \pm 4	40	13 \pm 2	20	11 \pm 1	15
Butacaine	19 \pm 2	40	10 \pm 2	25	10 \pm 1	25
Tetracaine	9 \pm 1	40	4 \pm 0.25	50	4.5 \pm 1	25
Dibucaine	6.5 \pm 0.7	40	2.8 \pm 0.2	40	2.8 \pm 0.5	25

minimize the influence on the results of secondary factors of inactivation, excretion, rate of distribution, etc.

Mice, guinea pigs, and rabbits were used for this study.

Carworth CF-1 female mice weighing between 16 and 20 grams were given injections at a constant rate of 50 mg./min. The volume of solution was less than 0.5 cc. and the administration was made into the tail vein. Guinea pigs used in this test weighed between 400 and 700 grams. The injections were made into a penile vein at a rate of 50 mg./min., the volume of solution being 0.1 to 0.5 cc. Albino rabbits weighing from 1.5 to 2.5 kg. were used. Injections were made into the marginal ear vein, the volume of the solution being 0.3 to 1.0 cc. and the duration of injection being 15 seconds. In all instances the solutions were prepared at the time of use, the dosage being calculated in mg. of base/kg. of body weight.

Manifestations of toxicity appeared immediately and death occurred in the mice and guinea pigs within 3 to 5 minutes. Rabbits died within 5 to 30 minutes. No late deaths among the animals that survived the immediate effects of the drugs were observed for any of the compounds.

The LD_{50} 's (5) of the various compounds are presented in table 1. It may be seen that inherent toxicity of the compounds increased in the following order as based on the LD_{50} data: procaine < neethesin \leq hexylcaine < cocaine < butacaine < tetracaine < dibucaine. Converting the LD_{50} data from mg./kg. to mols./kg. does not alter the comparisons qualitatively.

This information serves a limited purpose, as can be illustrated from common knowledge of the clinical use of procaine, neohesin and cocaine. Procaine and neohesin, which are of similar order of activity as compared to the other agents, are used more or less interchangeably although their inherent toxicity by this method differs considerably. Although cocaine, has the same order of inherent toxicity as does neohesin under these conditions, the former agent is contra-indicated for other than topical use because of its too frequent toxicity in clinical practise.

The *subcutaneous toxicity* of these agents is in a measure an integration of their rates of distribution, inactivation, and excretion with the inherent toxicity of the molecule, the last mentioned factor being an exaggeration of their pharmacodynamic effects.

TABLE 2

The subcutaneous toxicity of local anesthetic agents dissolved in aqueous solution and (for guinea pigs) in 1:50,000 solution of epinephrine hydrochloride

AGENT	LD ₅₀ , MG. (BASE)/KG. BODY WT.							
	Mice	No of animals	Guinea pigs				Rabbits	No. of animals
			Aqueous soln.	No of animals	Epinephrine soln.	No of animals		
Procaine.....	720 ± 64	70	353 ± 18	25	500 ± 146	25	595 ± 56	20
Neohesin.....	1140 ± 96	50	255 ± 33	20	305 ± 98	20	385 ± 45	25
Hexylcaine.....	1080 ± 213	60	146 ± 15	25	385 ± 40	25	164 ± 31	25
Cocaine.....	140 ± 21	40	27 ± 3	25	87 ± 7	25	70 ± 2	25
Butacaine.....	110 ± 12	70	39 ± 8	25	71 ± 7	20	30 ± 8	30
Tetracaine.....	64 ± 8	70	19 ± 6	20	78 ± 11	20	13 ± 2	20
Dibucaine.....	32 ± 3	90	18 ± 2	20	35 ± 4	30	7 ± 1	25

Mice, guinea pigs and rabbits of the same source and weight noted under *intravenous toxicity* were used for this study. The injections were made into the subcutaneous tissue of the flanks. Manifestations of toxicity appeared within a few minutes following the injections and death occurred within 5 to 30 minutes. Late deaths were not seen following the administration of any of these compounds.

It has been recognized for many years that the coadministration of epinephrine and a local anesthetic agent decreases the toxicity and prolongs the activity of the latter drug (6). The guinea pig subcutaneous toxicity studies were repeated with the addition of epinephrine in a final concentration of 1:50,000 to the local anesthetic solutions. The results of the subcutaneous toxicity studies have been summarized in table 2. In these data one sees more clearly than in table 1 a demarcation between the toxicity of procaine, neohesin and hexylcaine as a group, and the other compounds.

It was interesting to note the variation in the effect of epinephrine on the toxicity of the several agents. Whereas the coadministration of epinephrine

increased the subcutaneous LD_{50} for tetracaine over 4-fold, it increased the LD_{50} for neohesin by only 20 per cent. Under these conditions any difference in subcutaneous toxicity of hexylcaine and neohesin appears to be negated or reversed when they are administered in epinephrine solution.

Toxicity tests based on intermittent subcutaneous injections of the compounds were undertaken to determine whether the acute toxicity dosages of the agents could be exceeded if the drugs were administered in sublethal amounts at regular intervals over a period of time. Although the analogy is contestable, the problem may be thought to bear some relation to the clinical one wherein it is necessary to repeat the infiltration of an anesthetic agent once or several times in order to continue the desired effect without inducing manifestations of systemic toxicity.

The procedure used for these tests was as follows:

Ten albino rabbits weighing 1.5 to 2.5 kg. were selected for the tests on a single compound. Each rabbit was injected at 30 minute intervals with $\frac{1}{2}$ the subcutaneous LD_{50} of that agent. Two of the animals surviving four injections (the amount which when injected as a single dose killed 50 per cent of the animals (LD_{50})) were set aside to observe any delayed reactions. The survivors in the group were carried through six injections and of these two were put aside for observation. The remaining animals were injected twice more for a full set of 8 injections, or twice their LD_{50} , and were placed under observation.

Procaine, $LD_{50} = 600$ mg./kg.: Only the two animals set aside after the fourth injection survived the experiment. One died following the third, two following the fourth, and all the remaining animals died following the sixth injection which was $1\frac{1}{2}$ times the LD_{50} and which amounted to 900 mg./kg. All the animals lost their righting reflexes after the first injection but tended to regain them partially between injections.

Neohesin, $LD_{50} = 385$ mg./kg.: Two rabbits (20 per cent) died following the second injection ($\frac{1}{2}$ LD_{50}) and a third (30 per cent) following the fourth injection (LD_{50}). Two animals set aside after the fourth injection recovered uneventfully. Another rabbit (50 per cent of the number injected) died after the fifth injection. The two that were discontinued after the sixth injection survived and the remaining two animals recovered after the seventh (final) dose. Following the first three injections the animals tended to regain their righting ability between doses.

Cocaine, $LD_{50} = 70$ mg./kg.: Following the first injection, the animals became flaccid. Subsequent injections caused a loss of righting reflex which usually was regained before the next injection. There was little tendency toward loss of righting reflex after the fourth injection. All animals survived.

Tetracaine, $LD_{50} = 13$ mg./kg.: Four animals never lost their righting reflex. Three rabbits became flaccid with loss of righting reflex following the first injection. There was a general tendency toward recovery of righting ability between injections. All animals survived and the drug produced little visible effect after the fourth dose.

Butacaine, $LD_{50} = 30$ mg./kg.: One animal died following the second injection ($\frac{1}{2}$ LD_{50}). A general tendency toward recovery of the righting ability between

injections was evident following the first four injections. The remaining animals survived.

Dibucaine, $LD_{50} = 7$ mg./kg.: One animal died after two injections. Whereas there was no loss of righting reflex following the first injection, all the animals lost their ability to remain in an upright position following the second dose and remained in that state for the duration of the experiments. Those animals that were set aside for observation did not regain their ability to right themselves for over an hour following the injections. Two of the five animals given eight injections died during the night, presumably without recovering their ability to right themselves.

Hexylcaine, $LD_{50} = 164$ mg./kg. Some of the animals never lost their righting reflex, and those that did so tended to regain it between injections. The later

TABLE 3

A summary of the toxicity of local anesthetic agents when administered subcutaneously in doses of $\frac{1}{4}$ LD_{50} , repeated at intervals of 30 minutes

COMPOUND	MG./KG.	INTERMITTENT S.C. TOXICITY, RABBIT*				
		$\frac{1}{4}$ LD_{50}	LD_{50}	$1\frac{1}{2}$ LD_{50}	2 LD_{50}	Total
Tetracaine.....	13	0	0	0	0	0
Cocaine.....	72	0	0	0	0	0
Butacaine.....	30	10	—	—	—	10
Hexylcaine.....	(130)	0	0	0	0	0
	164	0	20	40	—	40
Neethesin†.....	385	20	30	50	—	50
Dibucaine.....	7	10	—	—	50	50
Procaine.....	600	0	30	100	—	100

* Toxicity is expressed as cumulative deaths, in percentage, among the animals that were given injections.

† This group of rabbits was carried through 7 ($1\frac{1}{4} \times LD_{50}$) instead of 8 injections ($2 \times LD_{50}$).

dosages produced little effect on the remaining animals. Four of the 10 animals died by the time the total dose was 1.5 times the LD_{50} . Thereafter none of the animals died. When the dosage on which the increments was based was reduced from 164 to 130 mg./kg. all the animals withstood the experiments.

These data have been summarized in table 3. The relative toxicities of the agents by this measure were almost the reverse of that seen for the acute toxicity data. Except for dibucaine the compounds injected in the lowest total dosage produced the least incidence of mortality even though the dosage in each instance represented the same fraction of their acute LD_{50} . Of the less acutely toxic compounds hexylcaine produced the lowest mortality figures in this experiment. Decreasing the basis for its dosage increments from 164 to 130 mg./kg. resulted in no deaths in the course of such a test. The significance of this type of experiment is uncertain. One might anticipate that the greatest mortality would accrue to the compound injected in the largest amounts, i.e. procaine. However in

clinical conditions requiring the repeated infiltration of effective concentrations more procaine would probably be required to produce a satisfactory effect than for the more potent compounds.

ACTIVITY STUDIES

Corneal anesthesia was selected as a suitable indicator for studying the efficacy of the various agents for topical anesthesia. It seemed hazardous to rely on any single criterion as an index of the relative activity of the compounds. Therefore both guinea pigs and rabbits were used as the test animals, and the experiment was designed to yield three indices of activity: 1) minimal concentration that

TABLE 4

The relative corneal anesthetic activity of local anesthetic agents

COMPOUND	CORNEAL ANESTHESIA IN GUINEA PIGS			CORNEAL ANESTHESIA IN RABBITS (USING SALINE WASH-OUT)			CORNEAL ANESTHESIA IN RABBITS (WITHOUT SALINE WASHOUT)		
	A.C. 100*	Dur. at 1%†	Conc. prod. dur. = 2% coc.‡	A. C. 100	Dur. at 1%	Conc. prod. dur. = 2% coc.	A.C. 100	Dur. at 1%	Conc. prod. dur. = 2% coc.
	per cent	min.		per cent	min.		per cent	min.	
Tetracaine§.....	$\frac{1}{4}$	52	$\frac{1}{2}$	$\frac{1}{4}$	98	$\frac{1}{4}$			
Butacaine.....	$\frac{1}{2}$	40	$\frac{1}{2}$	$\frac{1}{2}$	16	$\frac{1}{2}$	$\frac{1}{2}$	35	1
Hexylcaine.....	$\frac{1}{2}$	19	2	$\frac{1}{2}$	12	$\frac{1}{2}$	$\frac{1}{2}$	19	2
Cocaine.....	1	18	2	$\frac{1}{2}$	7	2	$\frac{1}{2}$	30	2
Neothesis.....	1	16	2	1	5	4	$\frac{1}{2}$	5	2
Procaine.....	4	0	7	8	0	16	4	0	8

* A.C. 100 = minimal conc. necessary to produce anesthesia in all animals.

† Duration of anesthesia when a one per cent solution was instilled.

‡ Percentage concentration that produced a duration of anesthesia equivalent to two per cent cocaine.

§ Corneal Anesthesia in Rabbits (without saline washout) was not determined.

would produce corneal anesthesia in all animals, 2) the duration of anesthesia when a one per cent solution was instilled, and 3) the concentration that produced the same duration of anesthesia as a two per cent solution of cocaine. As an added measure, the above indices of activity were applied to the rabbit tests wherein the application of the local anesthetic agent was 1) subsequently washed out with saline irrigation, or 2) allowed to remain without saline lavage.

The hair was clipped from around the eyes of the animals. The agent was instilled into only one eye, the other serving as a control for the return of the corneal reflex. In the case of the guinea pig test 0.5 cc. of the solution was applied to the eye and was allowed to remain or drain away. One cc. of anesthetic solution was applied to the rabbit eye. After one minute the eye was washed twice with saline or not at all, depending on the design of the test. Beginning at a concentration of 2 or 4 per cent, the concentration of solution was halved until the minimal anesthetic concentration was passed. The eyes were observed for evidence of irritation, mydriasis, cycloplegia, and loss of light reflex.

Table 4 summarizes the results of these tests. On the whole, hexylcaine was intermediate in its activity between butacaine and cocaine. The concentration

of hexylcaine necessary to produce anesthesia in all the rabbits (AC-100) was about the same as for butacaine. One and two per cent solutions of hexylcaine gave about the same duration of anesthesia as the corresponding concentrations of cocaine.

Tetracaine was the most active and procaine the least active compound. The tests with dibucaine were not completed since it was found to be irritating at the concentrations used for the greater number of these experiments. No irritation was noted when hexylcaine was instilled at these concentrations. The onset of anesthesia was almost immediate for all the agents.

Nerve block anesthesia was determined by using the sciatic nerve of the intact guinea pig as the test object, according to the method of Shackell (7).

The animals were restrained with all four limbs extended. The hair was shaved from the thigh of one hind leg and the pain response was determined by pinching the skin over the calf with a pair of blunted rat-toothed forceps. A $\frac{1}{4}$ cc. syringe was used and the injection was made by inserting the attached 26 gauge $\frac{1}{2}$ inch needle to a depth of 5 or 6 mm. at a point just behind the posterior curvature of the trochanter. The needle was withdrawn slightly and directed horizontally until the point lay on the trochanteric ridge. The needle then was slipped along the inner aspect of the trochanter until the point encountered the neck of the femur. The barrel of the syringe was raised slightly and the tip of the index finger was pressed between the anterior curvature of the trochanter and the vertebral column. The injection was made rapidly and the pressure was maintained for a few seconds after the needle was withdrawn.

The initial concentration of the drugs was 2 per cent (base). Subsequent concentrations were decreased by increments of $\frac{1}{2}$. The volume of solution was 0.1 cc. The experiments were designed to indicate 1) the actual percentage concentration necessary to produce anesthesia in all the animals tested (AC-100), 2) the duration of anesthesia at a concentration of one per cent, and 3) the percentage concentration that produced a duration of anesthesia equivalent to that induced by 2 per cent procaine.

The results of the sciatic nerve block experiments are presented in table 5. Again there was a certain inconsistency among the three indices of activity of the compounds. Hexylcaine was 4 to 8 times more active than was procaine by the several criteria.

The duration of hexylcaine anesthesia was unique since it was found that any concentration that would induce anesthesia produced a duration of effect exceeding that of 2 per cent procaine at lower concentrations in some animals than was necessary for local anesthesia in all animals.

At this point, table 5, we have introduced a figure for the therapeutic index or ratio of the compounds. The ratios were calculated by dividing the subcutaneous LD_{50} in guinea pigs by the actual amount of drug that was necessary to produce sciatic nerve block in all the animals (AC-100 in mg.). This has seemed a justifiable approach to the clinical margin of safety since the data are derived from a single species and the mode of administration is reasonably similar in the two tests.

The calculations of therapeutic ratio indicate a substantial margin of safety for hexylcaine. It is doubtful whether the index for tetracaine is significantly greater than for hexylcaine because of the tremendous difference an error in the

tetracaine data makes in the results. These data display prominently the relatively slight safety factor resident in the use of cocaine.

Spinal anesthesia studies may be considered a critical approach to the evaluation of the actual effect of local anesthetic agents on nerve conduction. Here the drugs are brought into intimate contact with conducting nerve tissue and diffusion is limited, for the most part, to the direction taken by the spinal cord and its nerve roots.

Rabbits were used for these studies since they could be worked with most conveniently in the large numbers that we considered necessary for accuracy. The method used was that of Bieter, McNearney, Cunningham and Lenz (8). A 21 gauge needle attached to a 1.0 cc. tuberculin syringe was inserted into the subarachnoid space, usually between the last

TABLE 5
The effect of local anesthetic agents on the sciatic nerve of guinea pigs

COMPOUND	SCIATIC NERVE BLOCK			
	AC ₁₀₀	Duration at 1%	% Conc. prod. dur. \approx 2% Procaine*	Therapeutic ratio†
	per cent	min.		
Tetracaine.....	$\frac{3}{4}$	112	$\frac{3}{4}$	608
Butacaine.....	$\frac{1}{2}$	54	$\frac{1}{2}$	312
Hexylcaine.....	$\frac{1}{2}$	57	$\frac{1}{2}$	584
Cocaine.....	$\frac{1}{2}$	30	$\frac{1}{2}$	54
Neothesis.....	$\frac{1}{2}$	18	$\frac{1}{2}$	510
Procaine.....	1	9	2	353

* Percentage concentration that produced a duration of anesthesia equal to the effect of two per cent procaine (12 minutes).

† Guinea pig $\frac{\text{sub. cut. LD}_{50}, \text{mg./kg.}}{\text{A.C.}_{100}, \text{mgm. (total)}} = \text{therapeutic ratio.}$

‡ An effective dose always produced a duration of anesthesia greater than for 2 per cent procaine. All 5 animals injected with $\frac{1}{2}$ per cent hexylcaine had a duration of anesthesia lasting 20 to 35 minutes (av., 25 min.) whereas a $\frac{1}{2}$ per cent solution did not induce anesthesia in any of 5 animals. Two per cent procaine induced local anesthesia of 12 minutes duration.

lumbar and first sacral vertebra. Aspiration always was attempted. We have been able to aspirate spinal fluid from an occasional animal but on the whole our findings in this regard confirm other reports concerning the paucity of spinal fluid in rabbits. When blood was aspirated the animal was discarded and was not used for spinal work. The dosage of all drugs used in this test was 0.02 cc. of a 1.0 per cent solution (calculated as base)/cm. of spinal length. The use of spinal length instead of weight for the calculation of dosage was introduced by Co Tui (9) and adopted by Bieter et al (8). The volumes ranged from 0.45 to 0.6 cc. and were injected at a rate of 1.0 cc./min.

Since the determination of duration of anesthesia and motor paralysis was somewhat subjective a team of three individuals was used. One operator kept a record of the identity of the rabbit and the drug, the dose based on spinal length, weight, and the time of onset of the injection. A second individual recorded spinal length, made the injection of the solution, the identity of which was unknown to him, and recorded any impressions regarding the animal's immediate response to the drug. A third member, who knew neither the identity of the drug nor the onset of the injection, recorded the time of recovery from anesthesia and motor paralysis. Later the data were assembled and summarized.

The results of these tests using 1.0 per cent solutions of all the drugs are presented in table 6. Tetracaine was the only compound whose duration of anesthesia and motor paralysis equalled or exceeded that of hexylcaine. As we shall see from the data presented in the next section, a one per cent solution of tetracaine administered according to this dosage schedule was the maximal tolerated concentration of that agent,⁸ whereas the minimal lethal concentration of hexylcaine was 4 per cent. The duration of action of hexylcaine was between 2 and 3 times longer than for procaine or neohesin. At a concentration of 1.0 per cent the onset of anesthesia for all the compounds was practically immediate. At this concentration all the animals injected with dibucaine died within a few minutes to an hour or so without recovery from the spinal anesthesia.⁸

Spinal toxicity studies were undertaken since the previous experiments gave no indication in most instances as to the approximation of the 1.0 per cent solution

TABLE 6

Duration of spinal anesthesia in rabbits when the local anesthetic agents were administered in volumes of 0.02 cc./cm. of spinal length

All solutions were one per cent (base) concentration in distilled water. The volumes of solutions ranged from 0.45 cc. to 0.60 cc. and were injected in the interspace between the fifth and sixth lumbar vertebrae at a rate of 1.0 cc./min

COMPOUND	NUMBER OF ANIMALS	AVERAGE DURATION OF ANESTHESIA	AVERAGE DURATION OF MOTOR PARALYSIS
		min \pm	min \pm
Tetracaine	6	193 \pm 84.3	251 \pm 95.9
Hexylcaine	18	55 \pm 24.2	73 \pm 27.5
Butacaine	15	41 \pm 13.3	56 \pm 17.1
Neohesin	12	23 \pm 6.0	34 \pm 6.0
Procaine	14	20 \pm 6.9	28 \pm 8.8
Dibucaine	5	5/5 dead	5/5 dead

to the minimal lethal concentration (MLC). Also it was considered desirable to study the incidence of paralysis and the manifestations of acute toxicity as the dosage of the various agents was increased.

Some of the animals were sacrificed one to several days after injection and the cords were examined grossly and microscopically. It was decided that the development or absence of bilateral motor paralysis over the course of time following the injection was a better evaluation of damage on a functional basis than we could make from an anatomical study. Co Tui, Preiss, Barcham and Nevin studied histologically the effect of local anesthetic agents on the spinal cord of cats and rabbits by an essentially similar technic (10). They concluded that "... In deciding which of these drugs is to be used as a preferable spinal anesthetic agent, the factor of tissue reaction may be eliminated from consideration."

The results of these studies are summarized in table 7. The minimal lethal

⁸ Bieter et al found the minimal lethal concentration of tetracaine to be 1.25 per cent, and of dibucaine, 0.4 per cent (8).

concentration (MLC) for *tetracaine* appeared to be between 1.0 and 2.0 per cent. Bieter et al. (8) killed one out of eight rabbits at a concentration of 1.25 per cent. The LC_{50} from our data was about 2.0 per cent and the LC_{100} was 4.0 per cent. It would seem reasonable to conclude that the data for duration of action of the compound in table 6 were obtained at the maximal tolerated concentration, 1.0 per cent.

The MLC for *hexylcaine* was 4.0 per cent. This represents a four-fold latitude beyond the concentration at which the data in table 6 were obtained. Evidently the spread of the toxicity data for this compound must be considerable since the same toxicity figure held for both the 4.0 and 8.0 per cent solutions.

TABLE 7
Spinal toxicity studies

The volume and rate of injection remained constant at 0.02 cc./cm. of spinal length at 1.0 cc./min. The concentrations of solutions were 1.0, 2.0, 4.0 and 8.0 per cent.

DRUG	ONE PER CENT		TWO PER CENT			FOUR PER CENT			EIGHT PER CENT	
	No. of animals	Duration anesthesia	Dead/total	Paralysis/alive	Duration anesthesia	Dead/total	Paralysis/alive	Duration anesthesia	Dead/total	Paralysis/alive
		min.			min.			min.		
Tetracaine.....	6	193	2/5	2/3	—	4/4				
Hexylcaine.....	18	55	0/5	3/5	77	1/5	3/4	126	1/5	4/4
Butacaine.....	15	41	0/5	3/5	57	2/5	3/3	102	5/5	
Neethesin.....	12	23	0/5	2/5	30	1/5	4/4	75	3/5	1/2
Procaine.....	14	20	0/5	0/5	32	0/5	0/5	45	0/5	2/5

Paralysis = bilateral motor paralysis.

The MLC for *neethesin* was 4.0 per cent, so that the same latitude pertained here as in the data for *hexylcaine*. However, the LC_{50} for *neethesin* was between 4.0 and 8.0 per cent whereas the LC_{50} for *hexylcaine* was greater than 8.0 per cent.

The MLC for *butacaine* was between 2.0 and 4.0 per cent, but since 4.0 per cent represents the LC_{50} , 2.0 per cent probably is the maximal tolerated dose and approaches the MLC. The LC_{100} for *butacaine* was 8.0 per cent.

It is evident that *procaine* was neither very active nor very toxic. No deaths occurred when the 8.0 per cent solution was injected, although toxic symptoms were noted when the 4.0 per cent concentration was employed.

In general the manifestations of toxicity for animals receiving the compounds in sublethal but toxic dosages were emprosthotonus or opisthotonus, clonic convulsions, and dyspnea. The animals that died acutely following a lethal dose had a generalized flaccid paralysis.

It is difficult to evaluate the data on residual or secondary paralysis following injection of the drugs. Only bilateral paralysis has been recorded in these data. The struggling of the animals and their long spinal cords made trauma a likely source of unilateral paralysis. It is evident that all the agents except *procaine*

produced a similar frequency of bilateral motor paralysis at concentrations greater than 1.0 per cent. The onset of paralysis was within a week and usually on the day or so following the injections. For an excellent report of the toxicity and activity of certain local anesthetic agents studied similarly one should refer to the work of Bieter, McNearney, Cunningham and Lenz (8).

DISCUSSION

The preclinical evaluation of new local anesthetic agents amounts to a compromise between toxicity and activity, wherein a subjective phenomenon, sensation, is measured objectively in lower animals and the results are projected to clinical safety and usefulness. The methods and presentation of data herein have been

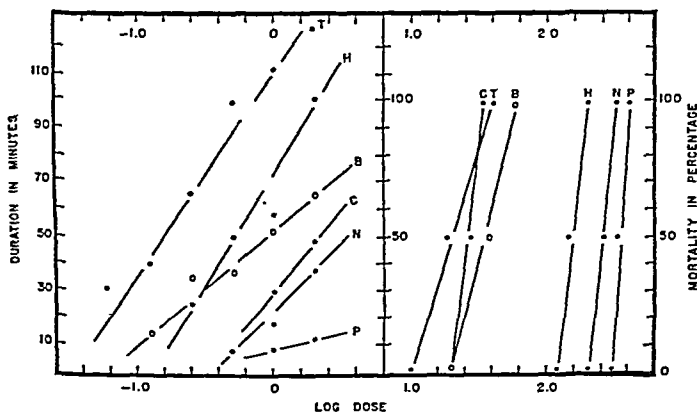


FIG. 1. A COMPARISON OF THE LOG DOSAGE-RESPONSE CURVES FOR TETRACAINE, HEXYLCAINE, BUTACAINE, COCAINE, NEOHESIN AND PROCAINE FOR THE DURATION OF SCIATIC NERVE BLOCK AND THE SUBCUTANEOUS TOXICITY DATA IN GUINEA PIGS

along conventional lines that will permit their ready comparison to earlier literature in the field as reviewed by Sollman (11), Hirshfelder and Bieter (12), Bieter (13) and others.

Perhaps another type of integration of these data may be helpful in evaluating the relative merits of the various compounds. While any number of comparisons may be made we have selected only one to aid in summarizing the data presented herein. In a single graph, figure 1, we have presented the log dosage-response curves for sciatic nerve block anesthesia and subcutaneous toxicity (mortality) in guinea pigs. Since in both instances the curves are reasonably linear their slopes and relative positions acquire significance. From these curves one may conclude that: 1) With increasing dosage one should get an incommensurately greater prolongation of activity with hexylcaine and tetracaine than for the other compounds. The coordinates for procaine and cocaine have been limited by the AC_{100} below, and the possible influence of salt concentration above, the dosages studied. 2) The margin of safety of hexylcaine was the greatest for any of the compounds as indicated by the proximity of the activity and toxicity curves.

3) Although the slope of the toxicity curves for all the compounds except tetracaine was essentially the same, they may be divided equally into two groups, (a) procaine, neohesin, and hexylcaine, and (b) butacaine, cocaine and tetracaine in order of increasing toxicity. Other comparisons between the data can be made. Tainter and Winter have pointed out the need for some such comparisons in the local anesthetic literature (14).

SUMMARY

Hexylcaine, 1-cyclohexylamino-2-propylbenzoate, has been compared with procaine, neohesin, cocaine, butacaine, tetracaine, and dibucaine on the basis of (a) toxicity (intravenous, subcutaneous, intraspinal and intermittent subcutaneous) and (b) activity (corneal, nerve block and spinal anesthesia) making use of mice, guinea pigs and rabbits. It appears that hexylcaine has (1) an order of toxicity comparable to that of procaine and neohesin, (2) an order of topical anesthesia comparable to cocaine and butacaine, (3) an order of nerve block anesthesia intermediate between butacaine and tetracaine, and (4) a duration of spinal anesthesia that was second to that of tetracaine at the maximal tolerated dose of the latter agent. These properties lead us to anticipate that hexylcaine may prove to be useful in the clinic.

REFERENCES

- 1a. HANCOCK, E. M., HARDY, E. M., HEYL, D., WRIGHT, M. E., AND COPE, A. C.: J. Am. Chem. Soc., 66: 1747, 1944.
- 1b. HANCOCK, E. M., AND COPE, A. C.: J. Am. Chem. Soc., 66: 1738, 1944.
- 1c. COPE, A. C., AND HANCOCK, E. M.: J. Am. Chem. Soc., 66: 1448, 1944.
- 1d. COPE, A. C., AND HANCOCK, E. M.: J. Am. Chem. Soc., 66: 1453, 1944.
- 1e. COPE, A. C., AND HANCOCK, E. M.: To be published.
2. KUNA, S., AND SEELE, A. O.: This Journal, 90: 181, 1947.
3. New and Non-official Remedies, 1947: American Medical Association, Chicago, Ill.
4. The Pharmacopoeia of the United States of America, Thirteenth Revision: Mack Printing Co., Easton, Pa. 1947.
5. EPSTINE, B., AND CHURCHMAN, C. W.: Ann. Math. Stat., 15: 90, 1944.
6. BRAUN, H.: Arch. f. klin. Chir., 69: 541, 1903.
7. SHACKELL, L. F.: Cur. Res. Anesth. Analges., 14: 20, 1935.
8. BIETER, R. N., MCNEARNEY, J. J., CUNNINGHAM, R. W., AND LENZ, O.: This Journal, 57: 221, 1936. Ibid., 57: 264, 1936.
9. CO TUI, F. W.: This Journal, 48: 223, 1933.
10. CO TUI, F., PREISS, A. L., BARCHAM, I., AND NEVIN M. L.: This Journal, 81: 209, 1944.
11. SOLLMAN, T.: A Manual of Pharmacology, W. B. Saunders Co., Philadelphia, Pa.
12. HIRSCHFELDER, A. D., AND BIETER, R. N.: Physiol. Rev., 12: 190, 1932.
13. BIETER, R. N.: Am. J. Surg., 34: 500, 1936.
14. TAINTER, M. L., AND WINTER, L.: Anesthesiol., 5: 470, 1944.

THE EFFECT OF SODIUM BICARBONATE ON THE RENAL EXCRETION OF SALICYLATE¹

FLORENCE WILLIAMS AND JACK R. LEONARDS

Department of Clinical Biochemistry, School of Medicine, Western Reserve University, Cleveland, Ohio

Received for publication March 9, 1948

Since the report in 1944 of Smull, Wegria, and Leland (1) showed that bicarbonate decreases the serum level of salicylate, there has been renewed interest in the advisability of administering sodium bicarbonate during salicylate therapy. Three possibilities were suggested for this effect of bicarbonate: (a) decreased intestinal absorption of the salicylate, (b) increased excretion of the salicylate, or (c) increased extracellular fluid volume.

That the decrease in blood salicylate level following bicarbonate administration is not due to impaired intestinal absorption was shown by Lester, Lolli, and Greenberg (2), who found that bicarbonate actually increased the rate of absorption of acetylsalicylic acid in humans. Investigations in this laboratory have shown that the administration of alkali has no effect upon the gastrointestinal absorption of salicylate in the rat. In these studies the procedure used was that described by Cori (3). Animals were intubated with 50 mgm. of sodium salicylate per 100 grams of body weight. After one hour the rats were sacrificed and the stomach and intestinal contents analyzed for the salicylate. The difference between the salicylate fed and the amount found in the gastro-intestinal tract after one hour represented the amount absorbed. The data on these animals were compared with the absorption data on rats receiving the same salicylate dosage plus 160 mgm. of sodium citrate and 25 mgm. of sodium bicarbonate. No significant difference in the rate of absorption was observed. Thus the low serum salicylate levels occurring after alkali administration must be due to some other factor than decreased intestinal absorption.

The action of bicarbonate to lower the serum salicylate level in dogs was studied in relation to the urinary excretion of the drug. Normal female dogs were found to tolerate an oral dose of 0.5 gram of sodium salicylate every three hours. With this course of administration the serum salicylate rose gradually to a level of about 60 mgm. per 100 ml. after two days. When the blood level had stabilized, the dog received 1.6 grams of sodium citrate and 0.25 gram of sodium bicarbonate in addition to the regular dose of sodium salicylate. The serum salicylate fell, within three to six hours to a level of 30 mgm. per 100 ml. During the next two days the serum level followed an irregular course of decline to a low of 15 mgm. per 100 ml. Upon cessation of citrate and bicarbonate administration, the salicylate level began slowly to rise again. The urinary excretion of salicylate showed a significant increase with the administration of bicarbonate, and this increase was of sufficient magnitude to account for the

¹Supported by a grant from the Ames Company, Elkhart, Indiana.

decreased blood level. An inverse relationship was shown between the serum salicylate level and the urinary pH. These findings are in agreement with a number of reports (2, 4, 5) which have since been made on this problem and are therefore not reported in detail here.

ANALYTICAL METHODS. The salicylate level in both plasma and urine was determined by the method of Brodie, Udenfriend, and Coburn (6) with some slight modification. Using this procedure, the salicylate determined in the plasma was the total amount of this ion found in the blood. The fraction of salicylate bound to plasma protein was determined by subjecting the plasma to ultrafiltration. This was carried out by placing the plasma in a small bag made of visking-cellulose sausage-casing, suspending the bag in a 15 ml. centrifuge tube and centrifuging for several hours.

To determine the total salicylate excretion in urine the conjugates were hydrolyzed by autoclaving 1 to 3 ml. of urine with 0.5 ml. of concentrated hydrochloric acid for 3 hours at twenty pounds pressure.

The creatinine level in the plasma and urine of the dogs was determined by the usual alkaline-picrate method (7). Urea level of the plasma and urine was determined by the method of Archibald (8).

PROCEDURE. Normal, healthy, female dogs, averaging 15 to 20 kgm., were fasted for 12 to 14 hours. They were anesthetized with nembutal and a continuous intravenous infusion apparatus set up in the vein of one of the fore-legs. Several control clearances were carried out, during which period the dog received 2.0 to 2.5 grams of sodium salicylate in physiological saline. Following this, the animal was given in addition to the salicylate, 100 ml. of a 10 per cent solution of sodium bicarbonate. Several additional clearance studies were then made. Blood samples were drawn from the femoral veins at the beginning and end of each thirty-minute period and the urine was collected by catheterization. When the animal received the initial dose of salicylate, it also was given subcutaneously 20 ml. of a 10 per cent creatinine solution. Thereafter, every hour during the course of the experiment, an additional 5 ml. of 10 per cent creatinine solution were administered by the same route. This schedule maintained the plasma creatinine level between 15 and 30 mgm. per 100 ml. which was suitable for renal clearance studies.

The procedure for studies on human subjects was much simplified, for the salicylate was taken by mouth, and the clearance was compared to that of urea.

Subjects were fasted for 12-14 hours before the experiments were begun. Each individual then received, over a period of three hours, 3 to 4 grams of salicylate, during which time, three, one-hour clearances were taken as controls. At the end of this time subjects were given 5 to 7 grams of sodium bicarbonate. One hour later, the subjects received a maintenance dose of 2 grams of sodium salicylate and another 5 or 7 grams of sodium bicarbonate. Two additional, one-hour clearances were then taken. At the beginning and end of each hour urine was voided as completely as possible and blood was drawn from the antecubital veins. Only those periods in which urine flow exceeded 2 ml. per minute were used in calculations presented in the tables. All results therefore are expressed in terms of maximum clearance.

EXCRETION

Results of these studies show that sodium bicarbonate increases the renal clearance of salicylate in dogs and humans. This is in agreement with reports of other investigators (2, 4).

In the Dog

Renal clearances in these experiments were calculated on the basis of the total salicylate excreted in the urine and the ultrafilterable salicylate in the plasma.

This is a valid calculation since at the present time the conjugated forms of salicylate, salicyluric acid and possibly the glucuronides, have not been demonstrated in the blood (2). As shown in table 1 the average renal clearance in the

TABLE 1

The effect of sodium bicarbonate on the renal excretion of salicylate in the dog

SUBJECT	PERIOD†	SALICYLATE IN PLASMA	SALICYLATE IN ULTRA-FILTRATE	pH OF URINE	URINARY SALICYLATE		RENAL CLEARANCE		URINE FLOW
					Before hydrolysis	After hydrolysis	Creatinine	Salicylate	
		mg./100 ml.	mg./100 ml.		mg./100 ml.*	mg./100 ml.*	ml./min.	ml./min.	ml./min.
Dog 1 Exp't 1	B	22	15	7.2	42	43	44	14	2.5
	B	24	16	7.0	48	62	44	19	2.3
	B	26	19	7.0	48	62	48	16	2.4
	A	23	18	8.1	175	220	49	70	5.8
	A	21	17	8.2	140	176	49	51	3.2
Dog 1 Exp't 2	B	15	10	5.7	4	15	49	7	2.5
	B	17	11	5.8	5	22	48	10	2.3
	A	16	11	8.1	135	149	63	82	5.5
	A	16	10	8.1	147	167	65	85	5.1
Dog 1 Exp't 3	B	30	26	5.3	—	21	42	4	2.0
	A	29	26	8.1	183	206	41	39	3.8
	A	27	24	8.4	179	218	41	45	3.7
Dog 2 Exp't 1	B	25	14	6.7	29	32	64	12	4.0
	B	25	14	7.0	39	44	65	15	4.1
	B	26	15	7.1	32	39	64	13	2.4
	A	24	14	8.3	153	188	68	65	4.2
	A	21	13	8.4	139	156	74	59	2.6
Dog 2 Exp't 2	B	32	19	6.9	24	45	57	11	2.8
	B	33	19	6.7	23	43	63	11	2.2
	A	31	19	7.7	84	146	74	46	6.2
	A	30	18	8.1	87	143	57	38	2.8
Dog 3 Exp't 1	B	38	21	5.6	4	32	66	7	3.3
	B	40	22	6.1	16	32	62	7	4.1
	A	35	20	8.2	157	191	48	47	4.5
	A	31	17	8.3	218	224	67	62	3.2
	A	24	12	8.2	133	142	64	56	2.9
	A	24	12	7.8	101	116	79	48	3.8

* Diluted to a volume of 5 ml. per minute before analysis.

† B indicates before alkali administration, and A indicates after alkali administration.

dog was 12 ml. per minute before bicarbonate and 85 ml. per minute after the intravenous injection of the sodium bicarbonate. Although the clearance of salicylate is to some degree affected by the volume of the urine flow, this large increase in the renal excretion of salicylate was not due to an increase in the

decreased blood level. An inverse relationship was shown between the serum salicylate level and the urinary pH. These findings are in agreement with a number of reports (2, 4, 5) which have since been made on this problem and are therefore not reported in detail here.

ANALYTICAL METHODS. The salicylate level in both plasma and urine was determined by the method of Brodie, Udenfriend, and Coburn (6) with some slight modification. Using this procedure, the salicylate determined in the plasma was the total amount of this ion found in the blood. The fraction of salicylate bound to plasma protein was determined by subjecting the plasma to ultrafiltration. This was carried out by placing the plasma in a small bag made of visking-cellulose sausage-casing, suspending the bag in a 15 ml. centrifuge tube and centrifuging for several hours.

To determine the total salicylate excretion in urine the conjugates were hydrolyzed by autoclaving 1 to 3 ml. of urine with 0.5 ml. of concentrated hydrochloric acid for 3 hours at twenty pounds pressure.

The creatinine level in the plasma and urine of the dogs was determined by the usual alkaline-picrate method (7). Urea level of the plasma and urine was determined by the method of Archibald (8).

PROCEDURE. Normal, healthy, female dogs, averaging 15 to 20 kgm., were fasted for 12 to 14 hours. They were anesthetized with nembutal and a continuous intravenous infusion apparatus set up in the vein of one of the fore-legs. Several control clearances were carried out, during which period the dog received 2.0 to 2.5 grams of sodium salicylate in physiological saline. Following this, the animal was given in addition to the salicylate, 100 ml. of a 10 per cent solution of sodium bicarbonate. Several additional clearance studies were then made. Blood samples were drawn from the femoral veins at the beginning and end of each thirty-minute period and the urine was collected by catheterization. When the animal received the initial dose of salicylate, it also was given subcutaneously 20 ml. of a 10 per cent creatinine solution. Thereafter, every hour during the course of the experiment, an additional 5 ml. of 10 per cent creatinine solution were administered by the same route. This schedule maintained the plasma creatinine level between 15 and 30 mgm. per 100 ml. which was suitable for renal clearance studies.

The procedure for studies on human subjects was much simplified, for the salicylate was taken by mouth, and the clearance was compared to that of urea.

Subjects were fasted for 12-14 hours before the experiments were begun. Each individual then received, over a period of three hours, 3 to 4 grams of salicylate, during which time, three, one-hour clearances were taken as controls. At the end of this time subjects were given 5 to 7 grams of sodium bicarbonate. One hour later, the subjects received a maintenance dose of 2 grams of sodium salicylate and another 5 or 7 grams of sodium bicarbonate. Two additional, one-hour clearances were then taken. At the beginning and end of each hour urine was voided as completely as possible and blood was drawn from the antecubital veins. Only those periods in which urine flow exceeded 2 ml. per minute were used in calculations presented in the tables. All results therefore are expressed in terms of maximum clearance.

EXCRETION

Results of these studies show that sodium bicarbonate increases the renal clearance of salicylate in dogs and humans. This is in agreement with reports of other investigators (2, 4).

In the Dog

Renal clearances in these experiments were calculated on the basis of the total salicylate excreted in the urine and the ultrafilterable salicylate in the plasma.

This is a valid calculation since at the present time the conjugated forms of salicylate, salicyluric acid and possibly the glucuronides, have not been demonstrated in the blood (2). As shown in table 1 the average renal clearance in the

TABLE 1

The effect of sodium bicarbonate on the renal excretion of salicylate in the dog

SUBJECT	PERIOD†	SALICYLATE IN PLASMA	SALICYLATE IN ULTRAFILTRATE	pH OF URINE	URINARY SALICYLATE		RENAL CLEARANCE		URINE FLOW
					Before hydrolysis	After hydrolysis	Creatinine	Salicylate	
		mg./100 ml.	mg./100 ml.		mg./100 ml.*	mg./100 ml.*	ml./min.	ml./min.	ml./min.
Dog 1	B	22	15	7.2	42	43	44	14	2.5
Exp't 1	B	24	16	7.0	48	62	44	19	2.3
	B	26	19	7.0	48	62	48	16	2.4
	A	23	18	8.1	175	220	49	70	5.8
	A	21	17	8.2	140	176	49	51	3.2
Dog 1	B	15	10	5.7	4	15	49	7	2.5
Exp't 2	B	17	11	5.8	5	22	48	10	2.3
	A	16	11	8.1	135	149	63	82	5.5
	A	16	10	8.1	147	167	65	85	5.1
Dog 1	B	30	26	5.3	—	21	42	4	2.0
Exp't 3	A	29	26	8.1	183	206	41	39	3.8
	A	27	24	8.4	179	218	41	45	3.7
Dog 2	B	25	14	6.7	29	32	64	12	4.9
Exp't 1	B	25	14	7.0	39	44	65	15	4.1
	B	26	15	7.1	32	39	64	13	2.4
	A	24	14	8.3	153	188	68	65	4.2
	A	21	13	8.4	139	156	74	59	2.6
Dog 2	B	32	19	6.9	24	45	57	11	2.8
Exp't 2	B	33	19	6.7	23	43	63	11	2.2
	A	31	19	7.7	84	146	74	46	6.2
	A	30	18	8.1	87	143	57	38	2.8
Dog 3	B	38	21	5.6	4	32	66	7	3.3
Exp't 1	B	40	22	6.1	16	32	62	7	4.1
	A	35	20	8.2	157	191	48	47	4.5
	A	31	17	8.3	218	224	67	62	3.2
	A	24	12	8.2	133	142	64	56	2.9
	A	24	12	7.8	101	116	79	48	3.8

* Diluted to a volume of 5 ml. per minute before analysis.

† B indicates before alkali administration, and A indicates after alkali administration.

dog was 12 ml. per minute before bicarbonate and 85 ml. per minute after the intravenous injection of the sodium bicarbonate. Although the clearance of salicylate is to some degree affected by the volume of the urine flow, this large increase in the renal excretion of salicylate was not due to an increase in the

urinary output which was often found after the injection of the large quantities of sodium bicarbonate.

It will be seen from table 1 that in the dog, under the conditions of our experiments and where the salicylate is injected intravenously, the greatest amount of salicylate excreted was not conjugated; 75 per cent to 90 per cent was excreted in the free form.

The creatinine clearances in the dogs were all within the normal range and indicate normal renal function. In the absence of bicarbonate the clearance of salicylate was much lower than that for creatinine, showing that salicylates are reabsorbed by the renal tubules after filtering through the glomeruli. However, with bicarbonate administration, the salicylate clearance did approach the creatinine clearance, pointing to an interference with the tubular reabsorption of salicylates. Beyer, Peters, Patch, and Russo (9) have reported that sodium bicarbonate interferes with the tubular reabsorption of a number of sulfonamides. The mechanism of the action of bicarbonate on the salicylate clearance in dogs may be a similar one.

In the Human

The effect of alkali on the renal clearance of salicylate in the human is similar to that observed in the dog, with certain important reservations. Sodium bicarbonate increases the salicylate clearance but the effect is not as striking as that in the dog. This may be due to the fact that the dogs received relatively larger amounts of bicarbonate and that the solution was injected intravenously. Further, more salicylate is bound to the plasma protein in the human than in the dog. The salicylate clearances in the human subjects were 35 to 115 ml. per minute in the absence of bicarbonate, but after the alkali was administered, rose to 50 to 173 ml. per minute.

As indicated in table 2 there was a large variation among the different subjects, but no part of the effect of the bicarbonate upon increasing the salicylate clearance can be attributed to a diuretic action, for, in direct contrast to its influence in the dog, sodium bicarbonate tended to suppress the urine flow in the human subjects of this study.

There was considerably less unconjugated salicylate excreted by the human than by the dog. Without alkali 30 per cent to 50 per cent of the total salicylate appeared in the urine in the free form, after administration of the alkali 75 per cent to 90 per cent was excreted in the free form. The increase in the renal clearance of salicylate, as the pH of the urine is increased, is due mainly to an increase in the excretion of unconjugated salicylate.

PLASMA BINDING

Since only the unbound fraction of salicylate in the blood can be filtered through the glomeruli, any change in the binding of salicylate to plasma protein will influence the excretion. One of the possible mechanisms by which bicarbonate could increase the salicylate excretion is by decreasing the bound fraction of the drug. In these clearance studies the salicylate content of ultrafiltrates of

plasma was determined with and without bicarbonate administration. The results of these analyses are shown in tables 1 and 2.

In the dogs the unbound salicylate constituted 60 per cent to 90 per cent of the total amount in the plasma, the per cent bound decreasing with increasing

TABLE 2

The effect of sodium bicarbonate on the renal excretion of salicylate in the human

SUBJECT	PERIOD†	SALICYLATE IN PLASMA	SALICYLATE IN ULTRAFILTRATE	pH OF URINE	URINARY SALICYLATE		RENAL CLEARANCE		URINE FLOW
					Before hydrolysis	After hydrolysis	Urea	Salicylate	
		mg./100 ml.	mg./100 ml.		mg./100 ml.*	mg./100 ml.*	ml./min.	ml./min.	ml./min.
JRL Exp't 1	B	21	3	6.4	19	36	85	84	7.0
	A	23	3	7.5	73	90	65	172	2.6
	A	25	5	7.0	64	83	76	134	8.0
JRL Exp't 2	B	18	2	5.5	9	21	84	90	5.1
	B	20	2	5.3	8	21	68	90	3.0
	A	28	5	6.8	42	64	67	116	3.3
JRL Exp't 3	B	17	2	6.6	11	28	81	113	3.0
	B	16	2	6.4	9	25	80	115	2.3
	B	24	4	6.1	11	30	59	67	2.0
	A	26	5	7.6	56	77	55	111	2.8
FMW Exp't 1	B	16	2	6.2	9	14	55	53	8.3
	B	16	2	5.5	5	11	46	46	4.4
	B	20	3	5.3	5	13	51	31	5.5
FMW Exp't 2	B	17	2	6.8	11	19	61	61	5.0
	B	15	2	6.6	12	19	62	72	5.9
	B	20	3	6.7	22	31	54	71	5.0
CTS Exp't 1	B	18	2	6.6	7	17	57	61	6.3
	B	25	4	6.2	9	19	43	37	8.5
	A	28	6	7.7	92	111	36	77	2.3
GDM Exp't 1	B	16	2	6.7	6	20	74	60	3.0
	B	16	2	6.6	5	16	82	83	10.0
	B	21	3	6.5	8	25	77	65	6.2
	A	19	3	7.6	36	56	73	132	3.2
	A	22	4	7.8	28	99	85	173	3.4

* Diluted to a volume of 8 ml. per minute before analysis.

† B indicates before alkali administration, and A indicates after alkali administration.

salicylate levels. The bound fraction showed no change in the presence of bicarbonate.

In the human subjects more salicylate was bound to plasma protein than in the dog. Here again bicarbonate had no significant effect on the amount of binding.

CONCLUSIONS

The administration of alkali to rats receiving sodium salicylate has no influence upon the gastro-intestinal absorption of this drug.

The oral administration of bicarbonate to dogs receiving sodium salicylate causes a decrease in the serum salicylate level. This decline can be accounted for by the large increase in the excretion of salicylate in the urine.

Sodium bicarbonate increases the clearance of salicylate four to nine times in a dog, to values approaching the glomerular filtration rate. In humans the action of bicarbonate upon the salicylate clearance points to an increase, but the effect is not nearly so marked. The salicylate clearance in human subjects is approximately equal to the urea clearance, but in the presence of administered bicarbonate the salicylate clearance rises above the urea clearance figure and also approaches the glomerular filtration rate.

Sodium bicarbonate has no significant effect upon the binding of salicylate by the plasma proteins of dogs or human. The large increase in the excretion of salicylates with bicarbonate cannot be accounted for by an increase in the ultra-filterable fraction of the salicylate in the blood.

Salicylates are excreted in the urine in the unchanged form and also as conjugates with glycine and glycuronic acid (10). In the dog, injected intravenously with sodium salicylate, 75 per cent to 90 per cent of the total salicylate is excreted in the free form. In the human subjects given salicylate orally, only 30 per cent to 50 per cent of the total salicylate is excreted in the free form. The administration of sodium bicarbonate mainly increases the output of the unconjugated salicylate.

REFERENCES

1. SMULL, K. WEGRIA, R., AND, Leland, J.: *J. A. M. A.* 125: 1173, 1944.
2. LESTER, D., LOLLI, G., AND GREENBERG, L.: *This Journal*, 87: 329, 1946.
3. CORI, C.: *J. Biol. Chem.*, 66: 691, 1925.
4. SMITH, P., GLEASON, H., STOLL, C., AND OGORZALEK, S.: *This Journal*, 87: 237, 1946.
5. WILLIAMS, F.: Dissertation for M.S., Western Reserve University, Cleveland, Ohio 1947.
6. BRODIE, B., UDENFRIEND, A., AND COBURN, A.: *This Journal*, 80: 114, 1944.
7. FOLIN, O., AND WU, H.: *J. Biol. Chem.*, 38: 81, 1919.
8. ARCHIBALD, R.: *J. Biol. Chem.*, 157: 507, 1945.
9. BEYERS, K., PETERS, L., PATCH, E., AND RUSSO, H.: *This Journal*, 82: 239, 1944.
10. KAPP, E., AND COBURN, A.: *J. Biol. Chem.*, 145: 549, 1942.

TOXICOLOGICAL AND PHARMACOLOGICAL STUDIES ON THE POWDERED STEM OF RYANIA SPECIOSA, A PLANT INSECTICIDE

SAMUEL KUNA AND RALPH E. HEAL

WITH THE TECHNICAL ASSISTANCE OF FRANK CUCHIE

*The Merck Institute for Therapeutic Research and the Merck Research
Laboratories, Rahway, N. J.*

Received for publication March 12, 1948

As early as 1922, investigators (1, 2) connected with the Brazilian Commercial Museum became interested in certain species of the genus "Ryania" (Flacourtiaceae). These investigators as well as Nakarai and Sano of Japan (3, 4) and Merz (5) of Germany were primarily interested in the highly toxic extraction products, ryanine and ryanitine, which they regarded as the active principles and on which physiological and toxicological studies were conducted. Preliminary chemical and pharmacological studies were also made by Serra (6). No mention however was made of possible insecticidal properties of these substances.

Through a cooperative research program established in 1939 between the Research Laboratories of Merck & Co., Inc. and the Department of Entomology of the New Jersey Agricultural Experiment Station and Rutgers University, the ground stem of the tropical plant *Ryania speciosa* Vahl, was found to be toxic to certain insects (7). In controlled field tests, conducted in two states, "Ryania" was found to be outstanding in activity against the European corn borer *Pyrausta nubilalis* (Hbn.) (8). It has also been found effective against the oriental fruit moth (9, 10) and several other species of insects of economic importance.

The purpose of this paper is to present the results of toxicological and pharmacological studies on the effect of the powdered stem of *Ryania speciosa*, in various animal species, together with comparisons with other known insecticides. The material used in this investigation, and referred to throughout this paper as Ryania powder, was undiluted stem of *Ryania speciosa* ground to the specification of 90 per cent through 200 mesh. This material was of proven insecticidal activity. Diluted Ryania powder will be referred to as Ryania dust. Dilutions of Ryania powder and cube powder (containing 5 per cent rotenone) were made with an insecticide grade of talc.

EXPERIMENTAL

SINGLE DOSE ACUTE ORAL TOXICITY

Mice, rats, chickens, guinea pigs, rabbits, dogs, and monkeys maintained on adequate diets with water ad libitum were used for this study. Ryania powder suspended in 5 per cent gum acacia was administered by stomach tube on the basis of mgm./kgm. body weight to all animals used in this investigation. In mice, rats, chickens and guinea pigs, a blunt No. 18 needle attached to a hypodermic syringe was used; to administer the suspensions to dogs, rabbits and monkeys, a soft rubber catheter was employed.

The results of these experiments (table 1) show wide variations in the LD50 values among the various species. This is evident also to a lesser degree among animals of the same species, which show a standard error as high as ± 90 mgm./kgm.

The early signs of toxicity after the administration of a lethal dose of a suspension of Rynia powder to mice, rats, guinea pigs, rabbits and dogs consist of inactivity and deep slow respiration followed by a general weakness. Subsequently the animals become semi-comatose and exhibit slow, deep, irregular breathing. In dogs, this stage is usually preceded by vomiting and defecation. Dogs, rabbits and guinea pigs occasionally exhibit slight tremors, while rats show

TABLE 1
Acute oral toxicity of Rynia powder

SPECIES	NO. OF ANIMALS	AVERAGE LD50	RANGE OF LD50*	STANDARD ERROR
		mgm./kgm.		mgm./kgm.
Dog.....	11	150		
Monkey.....	2	>400		
Rabbit.....	85	650	600-700	± 35
Mouse.....	285	650	450-1000	± 90
Rat.....	80	1200	1100-1500	± 70
Guinea pig.....	35	2500		
Chicken.....	60	>3000		

* Values obtained in different series of experiment.

slight and mice severe clonic convulsions. A deep comatose stage follows and death usually occurs within one hour.

COMPARISON OF ACUTE ORAL TOXICITY OF RYANIA POWDER AND OTHER INSECTICIDES

Mice weighing approximately 20 grams were used to determine the LD50 of various insecticides. At least 10 mice were employed per dose level and at least five levels of each sample were used. Each insecticide was administered by stomach tube in a 5 per cent gum acacia suspension.

It is evident from the data shown in table 2 that Rynia powder falls approximately at the mid point of the toxic range of other generally used commercial insecticides.

CHRONIC TOXICITY STUDIES WITH RYANIA POWDER

Rats, chickens, and guinea pigs were maintained on adequate diets containing various percentages of Rynia powder in a homogeneous mixture. Animals on the same diets, free of Rynia powder, served as controls. Food consumption and weights were recorded twice weekly.

The results of these tests (table 3) indicate that diets containing up to 1.0 per cent of the insecticide do not adversely affect the growth or food consumption

of the animals within a period of five months and that the effects of Ryania powder are not cumulative to an appreciable degree:

At autopsy, after this experimental period, no significant gross pathologic changes were observed in the chickens, guinea pigs or rats that had received 1.0 per cent or less of Ryania powder in the diet. Furthermore, no blood changes were evident in rats maintained on 1.0 per cent or less of Ryania powder in the diets. Rats dying from larger doses (2-5 per cent) of Ryania powder have shown hemorrhages in the pancreas and in the intestinal tract, pulmonary complications and pleural exudation.¹

TABLE 2
Comparative acute oral toxicity of insecticides (in mice)

SUBSTANCE	LD50
	<i>mgm./kgm.</i>
Nicotine Sulfate.....	15
Cube Powder*.....	95
Sodium Fluoride.....	100
Nicotine Dust (4% nicotine).....	500
Arsenic Trioxide.....	500
Ryania Powder.....	550
Arsenate of Lead.....	1000
DDT (Technical powder).....	2000
Natural Cryolite.....	10,000

* Containing 5% rotenone, 13% total resins.

COMPARATIVE CHRONIC TOXICITY STUDIES WITH RYANIA POWDER IN COMPARISON WITH OTHER INSECTICIDES

For this investigation, male rats were used. Various percentages of Ryania powder, "DDT" (Dichlorodiphenyl-trichloroethane) technical powder and cube powder (containing 5 per cent rotenone) were individually mixed with a normal diet on which the animals, 10 per group, were maintained. Rats on the normal stock diet served as controls. These experiments were continued for a period of twenty weeks.

These tests (table 3) indicate that under these experimental conditions Ryania powder is tolerated at a higher concentration than either "DDT" or cube powder (containing 5 per cent rotenone). During a five month period the concentrations tolerated in the diet without causing adverse effects were Ryania powder 1.0 per cent, "DDT" 0.01 per cent, and cube powder (containing 5 per cent rotenone) 0.025 per cent.

EXPOSURE TOXICITY

This investigation was designed to simulate extreme conditions that might be encountered in the field in the application of this insecticide. As the majority

¹ We are indebted to Dr. Charles W. Mushett and associates for the pathological and hematological data. A more detailed report on the pathological effects will be presented elsewhere.

TABLE 3

Chronic toxicity of Ryania powder and other insecticides
Insecticide in various amounts incorporated in the diet

SPECIES	PER CENT RYANIA POWDER IN DIET	NO. OF ANIMALS	AVG. WT. AT START	AVG. WT. AT END	PER CENT AVG. WT. GAINED	FOOD CON- SUMPTION	DUR. IN WEEKS	NO. DEAD
			<i>gms.</i>	<i>gms.</i>		<i>avg. in gms./day</i>		
Rats	0.025	20	307	411	33.9	—	20	2
	0.05	20	332	417	25.6	—	20	1
	0.1	20	334	431	29.0	—	20	4
	0.5	20	319	430	33.7	19.9	20	3
	1.0	20	321	427	33.0	22.4	20	3
	2.0	20	258	358	38.8	18.4	20	5
	5.0	20	235	157	-33.2	10.5	25 days	20
	Control	20	307	413	34.5	20.6	20	3
Chicks	0.5	10	56	1730	2990	55.2	20	0
	1.0	10	52	1641	3056	62.8	20	0
	Control	10	56	1804	3120	63.6	20	0
G. pigs	0.025	5	681	795	16.7	29.8*	20	1
	0.05	5	692	809	16.9	30.3*	20	1
	0.1	5	737	888	20.5	30.0*	20	0
	0.5	5	725	760	4.83	29.6*	20	2
	1.0	5	754	825	9.42	28.5*	20	1
	Controls	5	676	862	27.5	30.0*	20	2
Rats	% DDT IN DIET							
	0.005	10	232	346	49.1	18.3	20	0
	0.01	10	209	315	50.7	16.5	20	0
	0.025	10	173	336	94.2	15.7	20	2
	0.05	10	183	320	74.8	16.9	20	3
	0.1	10	178	294	65.1	13.3	20	3
	0.25	10	181	188	4.97	—	3	10
	0.5	10	176	156	-11.3	—	2	10
	1.0	10	175	144	-17.7	4.3	2	10
	5.0	10	179	149	-16.7	2	2	10
	Controls	10	172	325	88.9	17.7	20	0
Rats	% CUBE POWDER (CON- TAINING 5% ROTENONE)							
	0.025	10	221	311	40.7	18	20	1
	0.05	10	232	354	52.5	19.9	20	2
	0.25	10	181	295	63.0	15.7	20	7
	0.5	10	181	123	-32.1	12.6	8	8
	1.0	10	188	114	-39.3	10.4	3	10
	2.0	10	179	116	-35.2	—	3	10
	10.0	10	183	126	-31.1	0.46	2	10
	20.0	10	182	120	-34.1	0.63	2	10
	Controls	10	172	325	88.9	17.7	20	0

* Greens fed twice weekly not included.

of the experiments in this report were conducted on rats, this species was used again for this investigation. In addition, dogs which were found to be the most susceptible of several species to Ryania powder were included in the test.

In order to subject rats to a constant atmosphere of Ryania powder or to a spray of the aqueous suspension, special chambers were constructed. For the spray experiments, the chamber was made of 20 quart nickel plated tin cans in which a fan was installed to insure a constant circulation of air. A continuous spray of the Ryania powder suspension was then introduced. Similar chambers were used for the dust experiments in which the dust was kept constantly circulating. Since the dust chamber was practically a closed system, oxygen was added at the rate of 5.0 cc. per minute per rat. Soda lime was used for CO₂ absorption. Oxygen supplement was not necessary when an aqueous spray was used as air was continuously added. A fresh supply of Ryania powder, 2.5 grams, was added at 30 minute intervals to the dust chamber.

Exposure toxicity in rats: Four mature male rats, weighing approximately 200 grams each were exposed to a continuous spray of a 1.0 per cent aqueous suspension of Ryania powder at a rate of 5 cc. per minute for 8 hours per day, six days per week. Similarly 4 rats were exposed to a constant concentration of Ryania powder. For comparison, another group of 2 rats was exposed to 4 per cent nicotine dust.

The results of these tests indicate that rats exposed 8 hours daily for 22 days to Ryania powder or to a 1.0 per cent aqueous suspension of Ryania powder do not show any toxic signs. Rats tested similarly with 4 per cent nicotine dust died the first day at the end of a 4 hour exposure.

Exposure toxicity in dogs: The dust experiments with dogs were conducted in a special chamber having a volume of 64 cubic feet. The test material was continually added at a rate of 1.2 grams per minute by means of a dispenser. A blower was used to circulate the dust and to continually add fresh air to the chamber. Male dogs, weighing 10 to 14 kgm., were exposed to various concentrations of Ryania powder or cube powder (containing 4.7 per cent rotenone). The dispersing apparatus was adjusted so that 150 grams of dust would be introduced during a 2-hour period.

Both cube powder (containing 4.7 per cent rotenone) and Ryania powder are toxic to dogs exposed for 2 hours (table 4). Fifty per cent Ryania dust was found to be comparable to cube dust containing 1 per cent rotenone. Control dogs exposed to talc showed signs of irritation but no toxic signs.

EXCRETION STUDIES

Samples of urine from rats which had received 600 mgm./kgm. animal of a suspension of Ryania powder were assayed for the presence of the insecticidal principles of Ryania by a biological test. This test (11), developed in the Entomological Laboratory, Research & Development Division, Merck & Co., Inc., consists of an evaluation of feeding response from larvae of the webbing clothes moth on fabrics impregnated with the materials on test. Woolen fabrics were impregnated with the undiluted urine of the "Ryania"-dosed rats and subjected to the test in comparison with urine from rats on a standard diet and with samples of normal urine containing known amounts of the insecticidal principles. The tests showed that no detectable amount of "Ryania" principles was present in the urine from the rats dosed with a suspension of Ryania powder. The standard test showed that the lower limit of sensitivity of the test would permit the de-

tection of "Ryania" principles to the amount of the extractives from approximately 0.33 mgm. of Ryania powder per cc.

STUDIES WITH FIELD SPRAYED APPLES

Ten bushels of apples from field plots which had received 6 applications of a spray containing 6 lbs. of Ryania powder to 100 gallons of water, were obtained to determine their safety for consumption. The fruit was used to prepare a dust from the peels. This was done by drying and grinding the peels and finally sifting them to obtain a fine dust.

TABLE 4
Exposure toxicity in dogs

SUBSTANCE	NO. OF DOGS	NO. OF HOURS	SIGNS	REMARKS
Ryania Dust (50% Ryania Powder) 150 gm.	6	2	1 dog vomited; eyes and respiratory passages of all dogs irritated	1 dog died; others depressed; dogs normal after 24 hrs.
Cube Dust (Rotenone 1%) 150 gm.	6	2	3 dogs sick; eyes and respiratory passages of all dogs irritated	All dogs depressed; normal after 24 hrs.
Talc 150 gm.	6	2	Eyes and respiratory passages of all dogs irritated	Dogs not depressed
Ryania Powder 150 gm.	3	2	1 dog vomited; eyes and respiratory passages of all dogs irritated	1 dog died; 2 dogs depressed after 24 hrs.
Cube Powder (Rotenone 4.7%) 100 gm.	3	1½	2 dogs vomited; eyes and respiratory passages of all dogs irritated	2 dogs died; 1 depressed; normal after 24 hrs.

Although analysis (11) indicated that less than 6 parts per million of the insecticidal principles remained in the apple peels, this preparation was tested in both mice and rats. The mice were given single oral doses of 10 gm. per kgm. body weight, while rats were maintained for 13 weeks on a diet containing 1.0 per cent of this material (equivalent to 30 or more apples per day per man). No toxic or pathologic effects were evident in these animals.

DISCUSSION

Ryania speciosa stem powder administered in single oral doses to mice was found to be more toxic than "DDT". This may be partly due to the relative insolubility of "DDT" in aqueous suspensions since if administered in an oil solvent (12) the LD₅₀ is between 400-450 mgm./kgm. mouse. However, the repeated administration of "DDT" by addition to the diet indicates only 2 mgm. per rat can be tolerated for 28 weeks, while 200 mgm. of Ryania powder per rat

administered similarly was tolerated for the same length of time. This would indicate that "DDT" is markedly cumulative and Ryania powder relatively non-cumulative. An examination of table 3 substantiates this conclusion, in that with the insecticides chronically administered both "DDT" and cube powder cause 50 per cent of the animals to die at a far lower level than the acute oral LD50.

SUMMARY AND CONCLUSIONS

1. The acute oral toxicity of Ryania powder is 1200 mgm./kgm. in rats, 150 mgm./kgm. in dogs, more than 400 mgm./kgm. in monkeys, 650 mgm./kgm. in rabbits, 650 mgm./kgm. in mice, 2500 mgm./kgm. in guinea pigs and more than 3000 mgm./kgm. in chickens.
2. The signs of toxicity following administration of a lethal dose are: weakness, tremors, convulsions, coma and death.
3. The toxicity of Ryania powder to warm blooded animals compares favorably with that of other insecticides.
4. It is possible to maintain rats, chickens, or guinea pigs for at least five months on a diet containing 1.0 per cent or less Ryania powder. This concentration in the diet does not appear to produce symptoms of cumulative poisoning.
5. In chronic administration, Ryania powder is tolerated in larger amounts and for a longer period of time than either "DDT" or cube powder (containing 5 per cent rotenone).
6. Consideration of the demonstrated toxicity levels of Ryania powder in relation to the amounts of insecticidal residues normally encountered on forage and food crops indicates that a great safety factor exists for this insecticide.

REFERENCES

1. LECOINTE, P.: Bol. Ese. Chim. Ind. (Belem), 1: 43, 1930.
2. BRET, G.: Bol. Ese. Chim. Ind. (Belem), 1: 48, 1930.
3. NAKARAI, S., AND SANO, T.: J. Pharm. Soc. Japan, 48: 157, 1928.
4. NAKARAI, S., AND SANO, T.: Arch. Pharm., 272: 1, 1934.
5. MERZ, K. W.: Arch. Pharm., 268: 592, 1930.
6. Thesis by Quintero Serra, Central University of Venezuela.
7. HEAL, R. E., ET AL.: Unpublished data.
8. PEPPER, B. B., AND CARRUTH, L. A.: J. Econ. Entomol., 38: 59, 1945.
9. WHEELER, E. H.: J. Econ. Entomol., 38: 281, 1945.
10. WHEELER, E. H., AND LAPLANTE, A. A., JR.: J. Econ. Entomol., 39: 211, 1946.
11. HEAL, R. E., ET AL.: Unpublished data.
12. WOODARD, G., NELSON, A. A., AND CALVERY, H. O.: This Journal, 82: 152, 1944.

THE EFFECT OF VARIOUS SULFUR-CONTAINING COMPOUNDS ON ALPHANAPHTHYLTHIOUREA (ANTU) TOXICITY TO RATS

LEONARD KAREL¹ AND BERTRAM J. MEYER²

Toxicology Section, Medical Division, Army Chemical Center, Md.

Received for publication April 7, 1948

In an earlier communication, it was reported by Meyer and Karel (1) that of a large series of compounds tested for their effects on alphanaphthylthiourea poisoning in rats, only potassium iodide was prophylactically active, although not therapeutically so, whereas, cysteine, when administered at the time that ANTU was injected, delayed death for a few hours, without reducing mortality, and 1-thiosorbitol given concurrently with the ANTU significantly decreased mortality when tested against 2.0 median lethal doses of ANTU, but merely extended survival time when 2.5 LD₅₀'s were injected into rats. Moreover, no protection was achieved with thiosorbitol treatment initiated as little as one-half hour following the injection of the rodenticide. Since the only leads of therapeutic promise had been obtained with these two sulfur-containing compounds, it was decided to investigate other substances in which the sulfur was present in groupings comparable to the —SH or to the —C—C—SH of cysteine and thiosorbitol, and to the HO—C—C—S of thiosorbitol. Furthermore, since compounds

of the type
$$\begin{array}{c} \text{S} \\ \parallel \\ \text{N}-\text{C} \\ | \\ \text{S} \end{array}$$
 had not previously been tested, a substance having this group-

ing was included in the experiments. From the information to be obtained in studies such as those planned, it was hoped that there might be revealed fundamental data the pursuance of which would yield not only a drug effective against ANTU intoxication, but also insight into the mechanism of action of the rodenticide in causing pulmonary edema and pleural effusion. Therefore, as an extension of the previous work with sulfur compounds, studies were conducted with hydro-sulphosol;³ tertiary butyl, n-amyl, tertiary dodecyl, and benzyl mercaptans; 2-methylmercaptoethanol;⁴ thiodyglycol;⁴ 1,2-bis (2-hydroxyethyl-1-thio) ethylene;⁴ bis (2,2'-hydroxyethylmercaptoethyl) sulfide;⁴ and sodium diethyldithiocarbamate.

In addition, in view of recent reports extolling the efficacy of rutin (2-5) and of sodium salicylate (6) in decreasing capillary permeability, these two compounds and 5-iodoacetylsalicylic acid were also investigated.

¹ Present address: Research Grants Division, National Institute of Health, Bethesda, Maryland.

² Capt., M.C.

³ A mixture which reputedly contains the sulphydryl linkage and pentathionate ion in compounds of polysulfides and thiosulfates in aqueous solution.

⁴ Supplied by Dr. E. Emmet Reid and Dr. Benjamin Witten, Technical Command, Army Chemical Corps, Army Chemical Center, Maryland.

EXPERIMENTAL

Wistar albino rats weighing not less than 200 nor more than 300 gm. were used. Diet, periods of observation prior to and after treatment, methods of preparation of solutions and suspensions, and the procedures adopted for the evaluation of the test compounds were similar to those described in the earlier paper of this series (1). Controls were injected on each test day with the same intraperitoneal dose of ANTU as given to those animals subjected to treatment. Additional appropriate controls were run in all instances to eliminate the possibility of drug toxicity *per se* at the various dosage levels employed, although it was realized that at times potentiation effects might still occur. Usually, the drugs were given in maximal sub-lethal doses against 2 LD₅₀'s of ANTU.

With the exception of sodium salicylate, sodium diethyldithiocarbamate, and 1,2-bis(2-hydroxyethyl-1-thio) ethylene which are appreciably soluble in water and with the exclusion of bis (2,2'-hydroxyethyl-mercaptoethyl) sulfide, rutin, and 5-iodoacetylsalicylic acid given as suspensions in 20 per cent gum acacia, water, and 1 per cent methyl carboxylic cellulose, respectively, all other substances, being in the liquid state at room temperature, were injected in undiluted form.

RESULTS

Rutin was without detectable effect when injected in a dose of 500 mg./kg. intraperitoneally either 24 hours before or simultaneously with the injection of 2 LD₅₀'s (7.6 mg./kg.) of ANTU. Similarly, no beneficial effects were evident with sodium salicylate in a 300 mg./kg. dose administered either 24 hours before or at the time of injection of ANTU. Negative results were also obtained when 150 mg./kg. of 5-iodoacetylsalicylic acid were given 48 hours prior to the rodenticide or concurrently with the latter.

The results with the sulfur-containing compounds, arranged according to the chemical groups mentioned previously, are summarized in table 1. Hydro-sulphosol was totally ineffective in a dose of 2 ml./kg. injected simultaneously with the ANTU. Of the remaining compounds tested, thiodiglycol conferred definite, although not striking, protection, while diethyldithiocarbamate, 2-methylmercaptoethanol, 1,2-bis (2-hydroxyethyl-1-thio) ethylene, and bis (2,2'-hydroxyethyl mercaptoethyl) sulfide gave no protection. With the mercaptans, however, the findings were more encouraging. At equimolar doses (cf. table 1) corresponding to 0.5 ml. of the tertiary dodecyl mercaptan, which conferred no protection whatever, an unequivocal delay in onset of symptoms and in time of death could be attributed to the tertiary butyl, amyl, and benzyl compounds; and a definite reduction in mortality, to the butyl and the amyl mercaptans. As doses of the respective products were increased, the dodecyl again was ineffective, while the benzyl proved too toxic *per se*. The tertiary butyl and the amyl at 0.5 ml./kg., decreased the percentage of deaths from expected values of 85 and 77 per cent to 50 and 30 per cent, respectively, and further increased survival time.

Prophylactically, 0.5 ml. of amyl mercaptan injected intraperitoneally one hour prior to the injection of 2 LD₅₀'s of ANTU decreased mortality from 70 per cent to 20 per cent. When amyl mercaptan was given in divided doses (table 1), the protection was essentially no greater than with the single dose. Administered therapeutically as a single dose one hour after the injection of ANTU, the

TABLE 1
The effect of various sulfur-containing compounds on ANTU* toxicity in rats

NO.	COMPOUND	FORMULA	DOSE PER KG.†	TIME OF INJECTION (REFERRED TO ANTU)	NO. OF LD ₅₀ 's	MORTALITY		P†	APPROXIMATE MEAN SURVIVAL TIME (HOURS) OF ANIMALS DYING	
						Treated animals	Controls		Treated	Controls
1	Cysteine	$\text{COOH}-\text{CH}(\text{NH}_2)-\text{SH}$	1,000 mg.	Same	2‡	37/45 (82%)	33/35 (94%)	—	Increased— not calcu- lated**	—
2	L-Thioerbitol	$\text{HO}-\text{CH}(\text{NH}_2)-\text{SH}$ ($\text{CHOH}_2-\text{CH}_2\text{OH}$)	1,500 mg.	"	2	14/30 (47%)	27/30 (90%)	.0003	Increased— not calcu- lated**	—
3	Hydroaliphosol	$\text{C}_6\text{H}_5\text{SH}$	2.0 ml.	"	2	10/10 (100%)	10/10 (100%)	—	11.0	12.4
4	t-Butyl mercaptan	"	0.18 ml.††	"	2	6/10 (60%)	7/10 (70%)	—	18.2	15.0
5	"	"	0.5 ml.	"	2	10/20 (50%)	17/20 (85%)	.016	27.0	18.3
6	"	"	0.5 ml.	1 hr. later	2	6/10 (60%)	7/10 (70%)	—	20.2	15.0
7	n-Amyl mercaptan	$\text{C}_5\text{H}_{11}\text{SH}$	0.21 ml.††	Same	2	2/10 (20%)	7/10 (70%)	.028	17.8	13.0
8	"	"	0.5 ml.	1 hr. before	2	2/10 (20%)	7/10 (70%)	.038	14.0	11.6
9	"	"	0.5 ml.	Same	2	0/50 (0%)	23/50 (46%)	.0003	24.2	14.3
10	"	"	0.5 ml.	1 hr. later	2	8/20 (40%)	18/20 (90%)	.001	24.3	13.4
11	"	"	0.5 ml.	2 hrs. later	2	8/10 (80%)	10/10 (100%)	—	18.1	12.1
12	"	"	0.5 ml.	.25 ml.—asema .25 ml.—4 hrs. later	2	0/20 (0%)	14/20 (70%)	.012	27.8	14.9
13	"	"	0.5 ml.	Same	5	0/10 (0%)	10/10 (100%)	<.03	23.8	10.0
14	"	"	0.5 ml.	.25 ml.—asema .25 ml.—4 hrs. later	5	7/10 (70%)	10/10 (100%)	<.0001	17.7	10.0
15	"	"	0.5 ml.	Same	10	8/9 (89%)	10/10 (100%)	<.08	20.3	9.0
16	"	"	0.5 ml.	1 hr. later	10	10/10 (100%)	10/10 (100%)	—	13.9	9.0
17	t-Dodecyl mercaptan	$\text{C}_{12}\text{H}_{25}\text{SH}$	0.5 ml.††	Same	2	10/10 (100%)	7/10 (70%)	—	13.3	12.6
18	"	"	1.0 ml.	"	2	8/10 (80%)	9/10 (90%)	—	13.4	15.0
19	Benzyl mercaptan	$\text{C}_6\text{H}_5\text{CH}_2\text{SH}$	0.25 ml.††	"	2	7/10 (70%)	7/10 (70%)	—	18.6	12.6
20	2-Methyl mercaptoethanol	$\text{HO}-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_3$	1.5 ml.	"	2	7/10 (70%)	8/10 (80%)	—	15.6	14.1
21	Thiodiethyl	$(\text{HO}-\text{CH}_2-\text{CH}_2)_2\text{S}$	1.5 or 2.0 ml.	"	2	30/70 (51%)	65/70 (93%)	.0005	18.4	13.5
22	"	"	2.0 ml.†	"	2	12/23 (52%)	23/30 (77%)	.057	13.4	13.4
23	"	"	2.0 ml.	1 hr. before	2	17/30 (57%)	15/20 (75%)	—	25.3	18.6

24	1,2-Bis(2-hydroxyethyl)-1-thioethyleno	$(\text{HO}-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_2)_2$	1,000 mg.	Same	2	14/20 (70%)	13/20 (65%)	—	20.4	19.2
25	Bis-(2,3'-hydroxyethyl)-mercapto-ethylsulfido	$(\text{HO}-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-\text{S})_2$	500 mg.	"	2	15/24 (63%)	21/30 (70%)	—	15.5	15.3
26	Sodium diethyldithiocarbamate	$(\text{C}_2\text{H}_5)_2\text{N}-\overset{\text{S}}{\underset{\parallel}{\text{C}}}-\text{S}-\text{Na}$	200 mg.	"	2	9/10 (90%)	7/10 (70%)	—	13.9	12.8

* $(\text{C}_6\text{H}_5)_2\text{NH}-\overset{\text{S}}{\underset{\parallel}{\text{C}}}-\text{NH}_2$.

† Administered intraperitoneally except for No. 22 which was given intramuscularly.

‡ P = probability of difference occurring by chance, as calculated by the method of Lusk (7).

§ 1 LD₅₀ ± s.e. = $3.82 \pm \text{mg/kg}$; slope (b) = 4.30. At P = 0.01, mortality range at 2 LD₅₀'s = 50.5 to 99.3%.

** Previously reported (1).

†† Equimolar doses except for tertiary dodecyl mercaptan, which is ca. 1.2 times the others.

amyl mercaptan reduced mortality from 90 per cent to 40 per cent, again, in addition, producing a decided delay in onset of symptoms and in time of death. Even at 2 hours, the single dose treatment with the mercaptan appreciably prolonged survival time, although it no longer reduced mortality. Under comparable circumstances, tertiary butyl mercaptan was not as effective as amyl, merely prolonging survival time when administered one hour after the ANTU.

When 0.5 ml. of amyl mercaptan, administered at the time the rodenticide was injected, was tested against 19.1 and 38.2 mg./kg. of ANTU, corresponding to 5 and to 10 median lethal doses, the mortality was lowered from 100 per cent to 90 per cent and to 89 per cent, respectively, and the mean survival time of the animals dying was increased from the control values of 10 and of 9 hours to 23.8 and 20.3 hours. Divided doses (table 1) decreased the mortality from 100 per cent to 70 per cent. Although 0.5 ml. of amyl mercaptan administered one hour after 10 LD₅₀'s of ANTU gave no reduction in the number of deaths, mean survival time was again increased, from 9.0 to 13.9 hours.

DISCUSSION

In the previous report (1), reference has been made to the possible means by which thiosorbitol and cysteine may have conferred their protection. The currently gathered data shed additional light on mechanisms suggested earlier. A review of the findings reveals that of the various sulfur-containing substances studied, only the organic compounds possessing the —SH attached to carbon were at all effective. Compounds having no free —SH, with the exception of thiodiglycol which would be expected to hydrolyze readily and to yield a moiety having the sulphydryl group, were ineffective.

When comparisons were made of the structural formulas of the various compounds conferring protection to at least some extent, it was observed that the most effective of the compounds tested, n-amyl mercaptan, possesses two properties not simultaneously present in any of the others: (a) the —SH is attached to an unbranched aliphatic carbon chain; and (b) this chain is less than six carbon atoms in length. It is interesting in this regard that of the agents tested, the one closest to n-amyl mercaptan in effectiveness was the four-carbon compound, tertiary butyl mercaptan. While tests with the unbranched butyl mercaptan, etc., immediately suggest themselves, the authors have been compelled by circumstances to discontinue their studies with ANTU. It is their hope that others elsewhere will continue these experiments.

SUMMARY

1. n-Amyl mercaptan in an intraperitoneal dose of 0.5 ml./kg. was successful in delaying the onset of symptoms, increasing survival time, and reducing mortality when administered to rats simultaneously with the intraperitoneal injection of 2 LD₅₀'s of ANTU. When injected prophylactically one hour before ANTU was given, or therapeutically one hour following ANTU administration, the amyl mercaptan was approximately equally effective. Even against highly lethal doses of 5 and of 10 LD₅₀'s of ANTU, this compound decreased the mortality and markedly increased survival time.

2. Of other mercaptans tested, tertiary butyl was not quite as effective as the *n*-amyl; benzyl mercaptan was only slightly effective; and tertiary dodecyl mercaptan produced no beneficial results. Hydrosulphosol, reputed to contain free —SH groups, conferred no protection.

3. Experiments conducted with 2-methylmercaptoethanol, 1,2-bis (2-hydroxyethyl-1-thio) ethylene, and bis (2,2'-hydroxyethylmercaptoethyl) sulfide, were all negative. However, thioglycol caused a delay in the onset of symptoms, an increase in survival time, and a reduction in mortality when injected intraperitoneally in doses of 1.5 or 2.0 ml./kg. simultaneously with the injection of 2 LD₅₀'s of ANTU.

4. Sodium diethyldithiocarbamate, rutin, sodium salicylate, and 5-iodoacetylsalicylic acid were totally ineffective against 2 LD₅₀'s of ANTU.

REFERENCES

1. MEYER, B. J., AND KAREL, L.: *This Journal*, 92: 15, 1948.
2. GRIFFITH, J. Q., JR., COUCH, J. F., AND LINDAUER, M. A.: *Proc. Soc. Exper. Biol. and Med.*, 55: 228, 1944.
3. AMBROSE, A. M., AND DE EDS, F.: *Fed. Proc.*, 6: 306, 1947.
4. WILSON, R. H., MORTARROTTI, T. G., AND DE EDS, F.: *Fed. Proc.*, 6: 385, 1947.
5. ZFAS, H. S.: *Virginia Medical Monthly*, 74: 56, 1947.
6. COLESNICK, B., AND BEUTNER, R.: *Fed. Proc.*, 6: 314, 1947.
7. LUYKX, H. M. C.: *U. S. Nav. Med. Bull.*, 43: 1208, 1944; *Ibid.*, 44: 125, 1945.

THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON ALPHANAPHTHYLTHIOUREA (ANTU) TOXICITY TO RATS

BERTRAM J. MEYER¹ AND LEONARD KAREL²

Toxicology Section, Medical Division, Army Chemical Center, Md.

Received for publication April 7, 1948

It has been well established that differences in age, strain, and diet can cause striking variations in the susceptibility of rats to alphanaphthylthiourea (ANTU) (1-6). No mention, however, has been made of the effect of environmental temperature on the toxicity of this rodenticide. During the course of investigations, the purpose of which was to determine the mechanism of action of ANTU in causing acute pulmonary edema and pleural effusion (7, 8), it was noticed in this laboratory that rats were apparently more susceptible to the rodenticide during the hot summer months than at other times of the year. Also, sudden changes in temperature from day to day during this period, when the temperature in the vivarium was not controlled, frequently resulted in equally sudden changes in the LD₅₀ of ANTU. In order to obtain more detailed information concerning the variation in the median lethal dose of ANTU with changes in the environmental temperature, the following study was undertaken.

METHODS. Albino, Wistar, female rats weighing from 200 to 260 grams were used throughout this experiment. The animals were maintained for one month or more on the diet described in a previous communication (7). Injections of ANTU were given by the intraperitoneal route, with constant, non-toxic quantities (1 cc./kg.) of redistilled propylene glycol as the vehicle. The animals were divided into four groups and exposed, immediately after the injection of ANTU, to one of the following temperature ranges for a period of 16 or 24 hours (cf. table 1): (1) 36-38°F. (constant temperature chamber) (2) 46-50°F. (constant temperature chamber) (3) 68-75°F. (vivarium) (4) 87-90°F. (constant temperature chamber). Humidity was not controlled except with group (4), which was subjected to a relative humidity varying from 55-60 per cent during the experimental period. At the termination of the exposure period, the animals were removed to the vivarium and observed for a period of 5 days, at the end of which time the median lethal dose for each temperature range was determined by the method of Bliss (9).

RESULTS

The experimental data obtained are summarized in table 1. The median lethal doses and their standard errors for rats kept in environmental temperatures of 36-38°, 46-50°, 68-75° and 87-90°F. were, respectively, 1.90 ± 0.13 , 2.91 ± 0.24 , 4.03 ± 0.34 , and 1.23 ± 0.23 mg./kg. Thus, not only were the differences between the LD₅₀ at 68-75° and those at 36-38° and 87-90° highly significant,³ but

¹ Capt., M.C.

² Present address—Research Grants Division, National Institute of Health, Bethesda 14, Maryland.

³ Student's "t" test indicates that odds against the occurrence by chance of these differences are $> 1,744,000$ to 1.

significant differences ($P \geq 0.01$) occurred even between the not particularly widely separated ranges of 36–38° and 46–50°F, and 46–50° and 68–75°F. Mean survival time decreased as the LD_{50} decreased.

TABLE 1
Effect of environmental temperature on ANTU toxicity

DOSE mg./kg.	MORTALITY AT ENVIRONMENTAL TEMPERATURES (F)° OF			
	36–38*†	46–50*†	68–75° (Vivarium)	87–90*‡
8		6/6	20/22	
6		6/6	9/12	
4	6/6	15/18	11/18	6/6
3	11/12	4/6	4/18	6/6
2	11/18	1/12	1/18	5/6
1.5	1/6			
1	0/6			2/6
$LD_{50} \pm S.E.$ (mg./kg.)	1.90 ± 0.13	2.91 ± 0.24	4.03 ± 0.34	1.23 ± 0.23

* Humidity uncontrolled except for the 87–90° group, where the humidity was maintained at 55–60 per cent (rel.).

† Animals were kept in this environment for 24 hours before removing them to the vivarium.

‡ Animals were kept in this environment for 16 hours before removing them to the vivarium.

DISCUSSION

From the data presented in this report, it is apparent that the toxicity of ANTU to rats can be considerably influenced by environmental temperature. Of the four groups studied, those animals exposed to the vivarium temperature of 68–75°F. showed the greatest resistance to the rodenticide. It is especially interesting to note that an increase of 15 to 20°F. over the 68–75° range increased the toxicity of ANTU more than three times.

No attempt will be made to discuss the mechanisms that might be involved in causing the variations described. A review of the effect of body temperature and, to a lesser extent, environmental temperature on drug action has recently been published by Fuhrman (10).

The findings here reported emphasize the importance of including control animals with every experiment involving the use of ANTU, especially when the work is carried on in surroundings in which the temperature is apt to vary considerably from day to day. Because variation such as that described has long been suspected, it has been the policy of this laboratory to run a group of ten control rats, injected with ANTU alone, on each day that potential ANTU antagonists are tested.

SUMMARY

Changes in environmental temperature caused marked variations in the toxicity of ANTU to albino, Wistar rats. The importance of adequately controlled experiments involving the use of ANTU is emphasized by these findings.

REFERENCES

1. MACKENZIE, J. B., AND MACKENZIE, C. G.: *Proc. Soc. Exp. Biol. and Med.*, 54: 34, 1943.
2. RICHTER, C. P.: *J. A. M. A.*, 129: 927, 1945.
3. RICHTER, C. P., AND DIEKE, S. H.: *This Journal*, 83: 195, 1945.
4. RICHTER, C. P., AND DIEKE, S. H.: *Proc. Soc. Exp. Biol. and Med.*, 62: 22, 1946.
5. ROGERS, P. V.: *Proc. Soc. Exp. Biol. and Med.*, 63: 38, 1946.
6. LANDGREBE, F. W.: *Nature*, 157: 22, 1946.
7. MEYER, B. J., AND KAREL, L.: *This Journal*, 92: 15, 1948.
8. KAREL, L., AND MEYER, B. J.: *This Journal*. In press.
9. BLISS, C. I.: *Quart. J. Pharm. Pharmacol.*, 11: 192, 1938.
10. FUHRMAN, F. A.: *Physiol. Rev.*, 26: 247, 1946.

COMPARISON OF PRESSOR ACTION OF ALICYCLIC DERIVATIVES OF ALIPHATIC AMINES

EDWARD E. SWANSON AND K. K. CHEN

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis 6, Indiana

Received for publication April 8, 1948

In a previous report (1), a series of 39 aliphatic amines were described, and a conclusion was reached that compounds with an amino group on C_2 have a high index of pressor action. The present investigation was concerned with 11 alicyclic derivatives of ethyl and propyl amines. They were all synthesized and generously supplied by Doctors E. Rohrmann and G. F. Gilbert of our organic chemical division.

Cyclohexyl- and cyclopentyl-alkylamines have been subjects of study by Gunn and Gurd (2), Lands and his associates (3, 4), Zenitz, Macks, and Moore (5), and Marsh, Pelletier, and Ross (6). The sympathomimetic action of these substances has been generally established.

Of our series of compounds, as listed in table 1, 8 were basically 2-amino-propanes, and 3, amino-ethanes. They were all made into soluble salts—hydrochlorides or sulfates. For animal experiments, a 2 or 4 per cent solution was employed in each case.

Our primary purpose was to compare the pressor action of these compounds, and then to make an extensive study of the most active substance. The blood-pressure-raising property of each compound was evaluated in pithed dogs according to a method previously employed in this laboratory (1, 7, 8). Since all the active members of the series with one exception showed tachyphylaxis, to a more or less degree, after the first injection, groups of 3 to 7 animals for each amine were tested against epinephrine. Comparisons were made only with reference to the intensity of action, although the duration of action was also recorded in many experiments. A total of 49 dogs was used. All solutions were injected intravenously unless otherwise specified.

The results of the pressor action of these compounds are shown in the last column of table 1. The values might have been slightly different, had the same salts of the amines been used, be they hydrochlorides or sulfates. The differences, however, would have been so small that they could not have changed the order of activity.

With compounds Nos. 1 to 5 (table 1), it is apparent that substitution of one methyl group on amino N greatly increases the intensity of pressor action. Ethylation at the same position, on the other hand, decreases the activity. Substitution of 2 methyl groups on amino N (No. 2) similarly decreases the activity as well as the duration. The presence of a double bond between C_1 and the cyclopentenyl radicle, as in compound No. 4, also reduces the activity of the primary amine.

Regarding compounds Nos. 6 to 8, it may be pointed out that 2-amino-1-

cyclohexyl- is decidedly less active than 2-amino-1-cyclopentyl-propane. Methylation of the amino group in this case exerts an unfavorable influence on the pressor action; and further methylation to make it a tertiary amine abolishes almost 7/8 of the potency of the primary amine. Our results in this respect are at variance with those of Zenitz, Macks, and Moore (5), for they concluded that 2-methylamino-1-cyclohexyl- was more potent than 2-amino-1-cyclohexyl-propane.

The last 3 compounds, Nos. 9 to 11, are derivatives of ethane. When the cyclopentyl radicle is attached to the same C-atom as the amino group, the activity completely disappears; but if it is on the adjacent C-atom, the resultant compound is active (No. 9). The difference between this pair is unquestionably greater than a similar pair which Gunn and Gurd (2) investigated. They found

TABLE 1

Comparison of pressor activity of alicyclic derivatives of aliphatic amines in pilged dogs

COMPOUND				NO. OF DOGS USED	RANGE OF DOSES TESTED	MEAN EPINEPHRINE EQUIVALENT FOR 1 MILLIMOLE OF AMINE SALT
No.	Side chain	Stem nucleus	Salt used			
					mg.	millimole
1	2-Methylamino-1-cyclopentyl-	Propane	Hydrochloride	7	2-10	0.0053
2	2-Dimethylamino-1-cyclopentyl-		Hydrochloride	4	15-20	0.0018
3	2-Amino-1-cyclopentyl-		Sulfate	5	5-10	0.0028
4	2-Amino-1-cyclopentenyl-		Sulfate	5	6-12	0.0023
5	2-Ethylamino-1-cyclopentyl-		Hydrochloride	3	10-40	0.0021
6	2-Amino-1-cyclohexyl-		Sulfate	5	5-15	0.0021
7	2-Methylamino-1-cyclohexyl-		Hydrochloride	5	6-20	0.0014
8	2-Dimethylamino-1-cyclohexyl		Hydrochloride	4	20-60	0.0003
9	2-Amino-1-cyclopentyl-	Ethane	Sulfate	5	8-10	0.0014
10	2-Amino-2-cyclopentyl-		Sulfate	3	20-40	nil
11	2-Amino-1- <i>m</i> -methylcyclohexyl-		Sulfate	3	10	0.0010

that 2-amino-2-cyclohexyl-ethane was weaker than 2-amino-1-cyclohexyl-ethane, but not inactive. Methyl substitution on the cyclohexyl ring at meta-position (No. 11) does not improve the potency, but reduces it about 1/3. Compound No. 9, 2-amino-1-cyclopentyl-ethane, is less active than 2-amino-1-cyclopentyl-propane (No. 3), indicating the more favorable position of the amino group at the middle C-atom rather than at the terminal C-atom. Our observations are in agreement with those of Marsh and his co-workers (6).

All the cyclopentyl and cyclohexyl alkylamines of our series, except the tertiary amine No. 2 and the inactive compound No. 10, showed a long duration of action—longer than that of simple aliphatic amines. The effects of 2 compounds (Nos. 1 and 3) on blood pressure, following intravenous injection, persisted for about the same period of time as those of ephedrine. 2-Dimethylamino-1-cyclopentyl-propane is short acting.

Unlike simple aliphatic amines, the alicyclic derivatives are easily absorbed in the gastrointestinal tract, as evidenced by elevation of blood pressure following oral administration. To test this point more thoroughly, 2 amines (Nos. 1 and 3) were studied and compared with 2-amino-heptane, 2-amino-4-methyl-hexane, ephedrine, and amphetamine. Forty additional pithed dogs were used. All substances were given by stomach tube in the form of their soluble salts and in the doses as indicated in table 2.

The results of this study are shown in the last column of table 2. By comparing the average epinephrine equivalents of the 6 compounds, percentages were calculated, 2-methylamino-1-cyclopentyl-propane being taken as 100. Figure 1 shows the results of the 2 new compounds. In light of these data, ephedrine, in ease of absorption, is next to 2-methylamino-1-cyclopentyl-propane in the same doses by mouth. Amphetamine is less effectively absorbed as compared

TABLE 2

Comparison of pressor action by oral administration in pithed dogs

COMPOUND	SALT USED	NUMBER OF DOGS USED	RANGE OF DOSES STUDIED	RELATIVE ACTIVITY ON BLOOD PRESSURE
			mg. per kg.	per cent
2-Methylamino-1-cyclopentyl-propane	Hydrochloride	5	100-150	100
2-Amino-1-cyclopentyl-propane	Hydrochloride	7	100-200	74.2
Ephedrine	Sulfate	10	100-200	89.2
Amphetamine	Sulfate	8	100-200	39.3
2-Amino-heptane	Sulfate	5	200-400	26
2-Amino-4-methyl-hexane	Sulfate	5	200-400	20.5

with both 2-methylamino-1-cyclopentyl- and 2-amino-1-cyclopentyl-propanes. Only in large doses was there evidence of absorption of 2-amino-heptane and 2-amino-4-methyl-hexane, as measured by the rise of blood pressure.

FURTHER EXPERIMENTS WITH 2-METHYLAMINO-1-CYCLOPENTYL-PROPANE

Circulation. Since 2-methylamino-1-cyclopentyl-propane was found to be the most active of the 11 amines on blood pressure, it was decided to study this compound further prior to clinical trial. During the rise of blood pressure following intravenous injection of 2 mg., the nasal mucous membrane and accessory sinuses contracted, resulting in a drop of intranasal pressure recorded according to Jackson's method (9). This is demonstrated in figure 2.

It is now known that dibenamine, a synthetic product, has a sympatholytic action reversing the epinephrine response in blood pressure (10). Three more pithed dogs were employed to test the influence of dibenamine on the action of 2-methylamino-1-cyclopentyl-propane. In figure 3, it can be noted that dibenamine, injected beforehand, greatly reduced the pressor action of the new amine in the dose of 1 mg., but caused a slight fall followed by a rise of blood pressure in the dose of 5 mg. A pure reversal occurred with epinephrine in the same experiment.

The legs of 4 frogs were perfused according to Trendelenburg's method (11) with various concentrations of 2-methylamino-1-cyclopentyl-propane. A decrease in the rate of flow occurred with solutions of 1:2500 to 1:500—showing vasoconstriction. Greater dilutions were ineffective.

The hearts of 6 frogs were perfused with 2-methylamino-1-cyclopentyl-propane, according to the method of Howell and Cooke (12); and the hearts of 3 rabbits, by Langendorff's method (13). The results were similar in both species

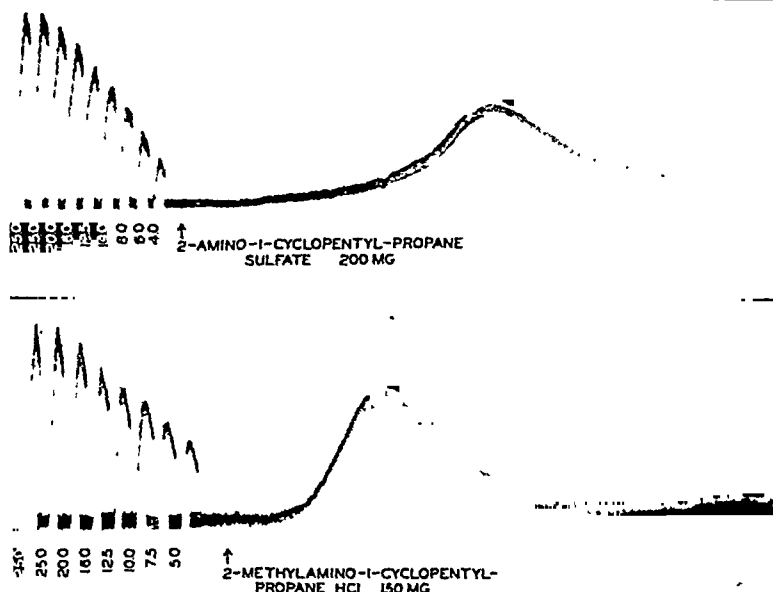


FIG. 1. ACTION ON BLOOD PRESSURE BY ORAL ADMINISTRATION

The blood pressure tracing on the top was made in a pithed dog, female, weighing 6.2 kg., and that at the bottom in another dog, male, weighing 7.4 kg. The numbers under the initial blood pressure curves designate the doses of epinephrine in $\mu\text{g.}$, injected intravenously. The 2 amines as indicated were both given by stomach tube.

of animals. Dilutions of 1:50,000 to 1:30,000 were ineffective, but concentrations of 1:20,000 to 1:2000 caused a decrease in heart rate, amplitude, and appearance of premature beats. At no time did stimulation occur.

Smooth Muscle Organs. 2-Methylamino-1-cyclopentyl-propane has the usual effects of sympathomimetic amines on smooth muscle organs. When a 1 per cent solution was dropped into albino rabbits' eyes dilatation of the pupil took place with preservation of light reflex. A 5 per cent solution of the same drug when instilled in the eyes of 4 human subjects produced mydriasis, lasting for 4 to 6 hours. Accommodation was not paralyzed. Observations were made in dim daylight. Slight stinging or burning occurred, but disappeared in a few seconds.

The new amine dilated bronchi of pithed dogs in the dose of 0.5 to 1 mg. per kg. given intravenously. The bronchial volume, recorded by Jackson's method (14), was constricted by pilocarpine hydrochloride immediately before the injection of 2-methylamino-1-cyclopentyl-propane.

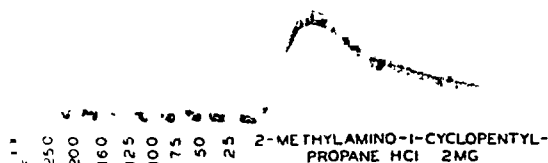


FIG. 2. ACTION ON NASAL VOLUME AND BLOOD PRESSURE

Dog, male, weighing 9.4 kg., was decerebrated and pithed. The top tracing is the nasal volume recorded by the Jackson technique. The numbers under the initial blood pressure curves indicate the doses of epinephrine in $\mu\text{g.}$, injected intravenously. The dose of 2-methyl-amino-1-cyclopentyl-propane HCl was also injected by vein.

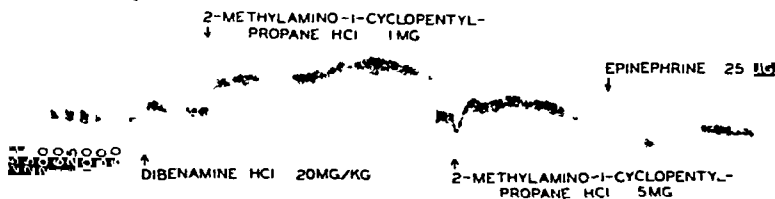


FIG. 3. EFFECT OF DIBENAMINE ON THE PRESSOR RESPONSE OF 2-METHYLAMINO-1-CYCLOPENTYL-PROPANE

Dog, female, weighing 7.8 kg., was decerebrated and pithed. The numbers under the initial blood pressure curves designate the doses of epinephrine in $\mu\text{g.}$, injected intravenously. Twenty minutes were allowed to pass after the administration of dibenamine by vein. There was a marked reduction of pressor action of 2-methylamino-1-cyclopentyl-propane. Pure reversal occurred with epinephrine.

On the isolated rabbit's small intestine dilutions of 1:10,000 to 1:5000 caused stimulation followed by inhibition, but those of 1:200,000 to 1:20,000 produced only stimulation. Both the isolated guinea pig's and rabbit's uteri were contracted with a 1:20,000 dilution or stronger solutions.

Central Nervous System. In view of Gunn and Gurd's report (2) that cyclohexyl-ethylamines exhibited a stimulating action on the central nervous system, tests were made with 2-methylamino-1-cyclopentyl-propane by subcutaneous injection in rats, according to the method of Schulte, Reif, Backer, Lawrence, and Tainter (15). Compared with amphetamine sulfate, our compound showed 1/40 the activity—being much less stimulating.

Toxicity. Acute toxicity was ascertained in albino mice by both intravenous injection and oral administration. The median lethal dose \pm standard error by vein was 41.6 ± 1.5 , and that per os, 168.7 ± 19.7 , mg. per kg. In comparison with ephedrine sulfate (16), 2-methyl-amino-1-cyclopentyl-propane is much more toxic; but in comparison with amphetamine, the results of which are not presented here, the new compound is approximately 1/6 as toxic.

Four groups of 5 rats each were subjected to continuous medication for 27 days. 2-Methylamino-1-cyclopentyl-propane was incorporated in food in the amounts of 0.01, 0.02, 0.05, and 0.1 per cent. The animals' body weights, hemoglobin, and erythrocyte, leucocyte, and differential counts were recorded weekly. All animals survived the experiment. They gained weight comparable to the untreated rats, except the group receiving 0.1 per cent of the drug in its food—it showed a slight inhibition of growth. The changes in hemoglobin, erythrocytes, and leucocytes were all within normal limits. Upon sacrifice at the end of the experiment, no pathological lesions could be detected by gross or microscopical examinations.

SUMMARY

1. A comparison of pressor action of 11 alicyclic ethyl and propyl amines has been made in pithed dogs.

2. The most active compound is 2-methylamino-1-cyclopentyl-propane. It has a long duration of action, and is effective in raising blood pressure in dogs by mouth.

3. 2-Methylamino-1-cyclopentyl-propane has the usual effects of sympathomimetic amines on smooth muscle organs.

4. 2-Methylamino-1-cyclopentyl-propane has an acute toxicity in mice by intravenous injection between that of ephedrine and amphetamine. Continuous administration of this compound in food to rats in concentrations varying from 0.01 to 0.1 per cent for a period of 27 days does not result in pathological changes of visceral organs.

Acknowledgment. The authors are indebted to Dr. Paul N. Harris and Messrs. C. E. Powell, R. C. Anderson, and Wallace C. Wood for their invaluable assistance in this investigation.

REFERENCES

1. SWANSON, E. E., AND CHEN, K. K.: *This Journal*, **28**: 10, 1946.
2. GUNN, J. A., AND GURD, M. R.: *J. Physiol.*, **97**: 453, 1940.
3. LANDS, A. M., LEWIS, J. R., AND NASH, V. L.: *This Journal*, **83**: 253, 1945.
4. LANDS, A. M., NASH, V. L., GRANGER, H. R., AND DERTINGER, B.: *This Journal*, **89**: 382, 1947.

5. ZENITZ, B. L., MACKS, E. B., AND MOORE, M. L.: *J. Am. Chem. Soc.*, 69: 1117, 1947.
6. MARSH, D. F., PELLETIER, M. H., AND ROSS, C. A.: *This Journal*, 91: 324, 1947.
7. SWANSON, E. E., SCOTT, C. C., LEE, H. M., AND CHEN, K. K.: *This Journal*, 77: 329, 1943.
8. SWANSON, E. E., STELDT, F. A., AND CHEN, K. K.: *This Journal*, 85: 70, 1945.
9. JACKSON, D. E.: *This Journal*, 31: 220, 1927.
10. NICKERSON, M., AND GOODMAN, L. S.: *This Journal*, 87: 167, 1947.
11. TRENDELENBURG, P.: *Arch. f. exper. Path. u. Pharmacol.*, 63: 161, 1910.
12. HOWELL, W. H., AND COOKE, E.: *J. Physiol.*, 14: 198, 1893.
13. LANGENDORFF, O.: *Arch. f. d. ges. Physiol.*, 61: 291, 1895.
14. JACKSON, D. E.: *This Journal*, 5: 479, 1914.
15. SCHULTE, J. W., REIF, E. C., BACKER, J. A., LAWRENCE, W. S., AND TAINTER, M. L.: *This Journal*, 71: 62, 1941.
16. CHEN, K. K., AND SCHMIDT, C. F.: *Medicine*, 9: 1, 1930.

CHEMOTHERAPEUTIC STUDIES ON A SERIES OF DITHIOCARBAMATES AND THEIR BISMUTH DERIVATIVES¹

ANDRES GOTH AND FABIAN J. ROBINSON

Department of Physiology and Pharmacology, Southwestern Medical College, Dallas, Texas

Received for publication April 13, 1948

Earlier studies by one of us (1, 2) have shown that the antibacterial activity of antibiotics of the aspergillic acid group was increased markedly by the addition of bismuth. Aspergillic acid showed a tendency to form complexes with certain metals and its bismuth complex possessed a considerable antibacterial activity. Since it seemed possible that other metal reagents would act similarly, a number of them were examined. During the course of this study, it was found that certain dithiocarbamates, which are widely used as precipitants of metals, are active antibacterial substances and that certain bismuth dithiocarbamates are much more active than the compounds from which they derived.

MATERIAL AND METHODS. The dithiocarbamates were obtained from the sources indicated in table 1. They were crystalline solids, with the exception of sodium dimethyl dithiocarbamate which was obtained in a 30 per cent aqueous solution. The bismuth derivatives were prepared by mixing aqueous solutions of equimolar quantities of a dithiocarbamate and bismuth ammonium citrate (Mallinckrodt). The precipitated bismuth dithiocarbamates were washed repeatedly with water and dried in air. The compounds were insoluble in water, very soluble in chloroform and pyridine and slightly soluble in ether.

Studies in vitro. The various dithiocarbamates were dissolved in water. The bismuth derivatives were dissolved in pyridine (2 per cent solution) and dilutions were made in water. Difco nutrient broth was used in all tests. Citrated human plasma was added to the broth in the tests indicated in table 1. The test organisms were stock cultures except where otherwise indicated. The susceptibility of the strain of *Staphylococcus aureus* used was approximately the same as that of the FDA strain of the same species. Growth inhibition was judged by the lack of visible growth in the test cultures.

Protection experiments in mice. Bismuth diethyldithiocarbamate was suspended in 3 to 5 per cent gum acacia or propylene glycol. Gum acacia was preferable, for it produced little or no local irritation. The drug was injected intramuscularly in a 10 per cent suspension at the times indicated in table 2. The infecting organism was the R1 strain of *Pneumococcus* type 1 from the Bureau of Laboratories, New York City Health Department. The infecting dose consisted of 0.2 cc. of the indicated dilution of an 18-hour culture. Injection was made intraperitoneally.

RESULTS. The inhibitory activity of 9 dithiocarbamates and 4 bismuth dithiocarbamates is indicated in table 1. The most active member of the dithiocarbamate series was the dimethyl derivative. Plasma tended to counteract the effects of all compounds, but sodium dimethyldithiocarbamate retained considerable activity in the presence of 10 per cent plasma. Bismuth derivatives of dimethyldithiocarbamic acid and diethyldithiocarbamic acid were 5 and 20 times

¹ A preliminary report of this work appeared in *Federation Proc.*, 7: 223, 1948.

as active, respectively, as the sodium salts in broth, and each retained considerable activity in 10 per cent plasma.

A number of protection experiments were carried out in mice using the compounds which showed unusual activity *in vitro*. Pneumococcus type 1 was used as the infecting agent. Studies *in vitro* have shown that this organism is as susceptible to the various drugs used as is the staphylococcus. In a preliminary experiment (not included in table 2), sodium dimethyldithiocarbamate failed to protect mice infected with a 10^{-3} dilution of an 18-hour culture of pneumococcus (type 1). Fifty mgm. of the drug per kgm. was injected intraperitoneally every

TABLE 1

Activity of dithiocarbamates and their bismuth derivatives against Staph. aureus

DRUG	SOURCE*	MINIMAL GROWTH INHIBITORY CONCENTRATIONS	
		In broth	In broth + 10% plasma
		mgm. 100 cc.	mgm. 100 cc.
Sodium dimethyldithiocarbamate.....	DP	0.1	1
Sodium diethyldithiocarbamate.....	EK	0.4	>5
Sodium bis(2-Hydroxyethyl)-dithiocarbamate.....	B	5	>5
Sodium ethyl-sec.-butyldithiocarbamate.....	EL	>5	>5
Sodium morpholyldithiocarbamate.....	EL	2	>5
Sodium ethylisoamyldithiocarbamate.....	EL	>5	>5
Sodium phenylethyldithiocarbamate.....	DP	5	>5
Sodium dibenzylldithiocarbamate.....	DP	4	>4
Piperidinium 1-piperidyldithiocarbamate.....	DP	0.2	>5
Bismuth dimethyldithiocarbamate.....		0.02	0.2
Bismuth diethyldithiocarbamate.....		0.02	0.2
Bismuth 1-piperidyldithiocarbamate.....		0.1	1
Bismuth phenylethyldithiocarbamate.....		1	5
Bismuth and ammonium citrate USP IX.....	M	>5	>5
Sodium bismuth thioglycollate.....	PD	>5	>5

* DP = E. I. du Pont de Nemours and Company; EK = Eastman Kodak Company; B = Baker Chemical Company; EL = Eli Lilly and Company; M = Mallinckrodt Chemical Works; PD = Parke, Davis and Company.

3 hours. Eighteen mice were used in both the treated and the untreated control groups.

In 3 experiments reproduced in table 2, the effect of intramuscular injections of bismuth diethyldithiocarbamate was studied in experimental infections due to pneumococcus (type 1). Since the drug is very insoluble, treatment was started 2 or 7 days prior to the infection. The results indicate that this drug, when injected intramuscularly in a dose of 500 mgm. per kgm., is capable of protecting mice against an otherwise fatal pneumococcal infection.

In the experiments in which a single injection of the drug was given, the observed differences between per cent survivors in the treated and in the untreated groups were less than 2 standard deviations of the differences. On the other

TABLE 2

Activity of bismuth diethyldithiocarbamate against pneumococcus type 1 infection in mice

EXP. NO.	GROUPS	DILUTION OF CULTURE	NO. OF MICE	NUMBER OF DEATHS							MEAN SURVIVAL TIME	SURVIVORS	
				Days after infection								No.	%
				1	2	3	4	5	6	7			
1	Untreated	5×10^{-3}	14	5	8	0	0				hours 30	1	7
	Untreated	5×10^{-5}	6	0	6	0	0				38	0	0
	Treated*	5×10^{-3}	14	3	6	0	1				37	4	28
2	Untreated	10^{-3}	18	0	18	0	0	0	0	0	32	0	0
	Untreated	10^{-5}	6	0	5	1	0	0	0	0	36	0	0
	Treated†	10^{-3}	19	0	6	3	2	1	0	2	66	5	26
3	Untreated	10^{-3}	19	0	12	5	0	0	0	0	43	2	10.5
	Untreated	10^{-5}	6	0	4	1	0	0	0	0	42	1	16.6
	Untreated	10^{-7}	6	0	0	0	0	0	0	0	—	6	100
	Treated‡	10^{-3}	20	0	7	3	1	3	0	0	63	6	30
	Treated‡	10^{-3}	20	0	5	2	1	0	0	1	63	11	55

* Single intramuscular injection of 500 mgm. per kgm., 2 days prior to infection.

† Two intramuscular injections of 500 mgm. per kgm. each, 7 days and 2 days prior to infection.

‡ Single intramuscular injection of 500 mgm. per kgm., 7 days prior to infection.

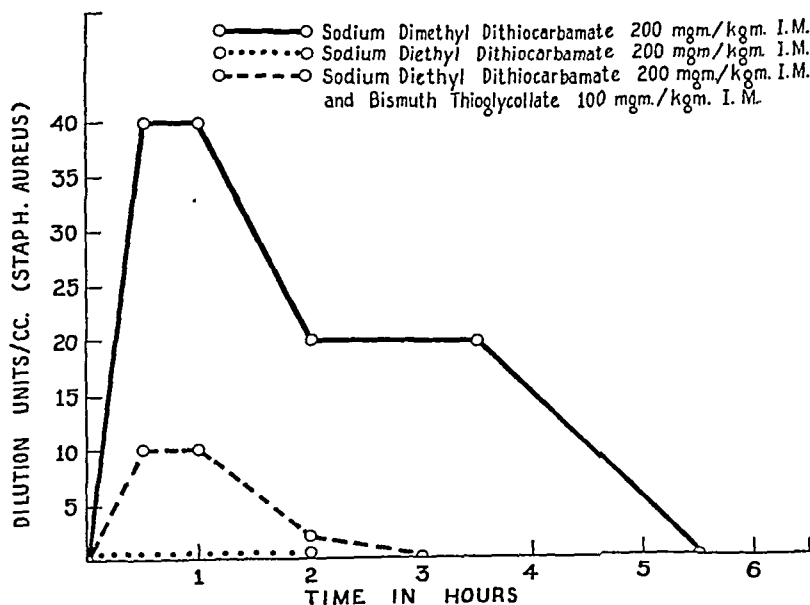


FIG. 1

hand, where 2 injections were given the observed differences were 31 and 73 per cent greater, respectively, than 2 standard deviations of the differences.

The antibacterial spectrum of bismuth diethyldithiocarbamate appears to be wide. *Brucella melitensis*, *Mycobacterium tuberculosis*, strain 607 (American Type Culture Collection), and *Escherichia coli* are inhibited by a concentration as low as 0.1 mgm. per 100 cc.

The acute toxicity of sodium dimethyldithiocarbamate and of piperidinium 1-piperidyldithiocarbamate has been studied by Dieke *et al.* (3). The intraperitoneal MLD for rats according to these authors is greater than 500 mgm. per kgm. The same dose of sodium diethyldithiocarbamate failed to kill any of 10 mice when injected by us intraperitoneally. The acute toxicity of bismuth diethyldithiocarbamate is low when given intramuscularly as indicated by our protection experiments in mice. In contrast to the activity of the bismuth derivative, the iron and copper salts of diethyldithiocarbamic acid were less active against the staphylococcus than was the sodium salt.

DISCUSSION. A review of the literature reveals numerous references to the fungicidal properties of certain dithiocarbamates, summarized by Tisdale and Flenner (4). We have been unable to find studies on dithiocarbamates as possible antibacterial chemotherapeutic agents or on the potentiating effect of bismuth.

Our studies reveal numerous similarities between aspergillic acid and the dithiocarbamates. Both tend to precipitate metals, such as iron, copper, and bismuth. The antibacterial activity of both is decreased by iron and is potentiated by bismuth. The findings with the present series of compounds strengthen the hypothesis put forward in a previous communication (5) in which it is suggested that the antibacterial activity of certain compounds may be due to interference with metal-catalyzed enzymatic reactions. Kubowitz (6) has shown that diethyldithiocarbamate is a powerful inhibitor of catechol oxidase, a copper enzyme.

The physical properties of bismuth dithiocarbamates would tend to make them more suitable for the treatment of chronic experimental infections and such studies are in progress.

SUMMARY

Certain dithiocarbamates were found to possess antibacterial activity. Certain bismuth derivatives were much more active than the parent compounds. Bismuth diethyldithiocarbamate was capable of protecting mice against an otherwise fatal infection caused by pneumococci (type 1).

REFERENCES

1. GOTH, A.: Science, 104: 330, 1946.
2. GOTH, A.: Federation Proc., 6: 333, 1947.
3. DIEKE, S. H., ALLEN, G. S., AND RICHTER, C. P.: This Journal, 90: 260, 1947.
4. TISDALE, W. H., AND FLENNER, A. L.: Indust. & Engin. Chem. (Indust Ed.), 34: 501, 1942.
5. GOTH, A.: J. Lab. & Clin. Med., 30: 899, 1945.
6. KUBOWITZ, F.: Biochem. Ztschr., 292: 221, 1937.

ADRENERGIC BLOCKING DRUGS

I. COMPARISONS OF EFFECTIVENESS IN DECREASING EPINEPHRINE TOXICITY IN MICE¹

EARL R. LOEW AND AUDREY MICETICH

Department of Pharmacology, University of Illinois College of Medicine, Chicago

Received for publication April 14, 1948

The majority of drugs known to exert adrenergic blocking activity were found to decrease the toxicity of epinephrine in mice. Comparisons were made of the effectiveness of known adrenergic blocking drugs and new synthetic compounds (1, 2, 3) in diminishing epinephrine toxicity. Compounds other than adrenergic blocking drugs were also studied in order to determine whether the ability to reduce epinephrine toxicity was largely restricted to adrenergic blocking agents.

METHODS. Drugs, in a volume of 0.1 cc. of water, were administered orally to groups of 20 mice (19-21 grams; non-fasted; both sexes). Two hours later the mice were injected intraperitoneally with 14.4 mgm./kgm. of epinephrine hydrochloride (equivalent to 12.0 mgm./kgm. of epinephrine base) which sufficed to kill 67.0 ± 1.53 per cent of 40 groups of 20 control mice (saline treated). In mice pretreated with non-toxic doses of drugs, decrease of this mortality would suggest antagonism of the effect of epinephrine. Drugs were considered effective if the mortality within a treated group was decreased significantly from the mortality occurring concurrently in a group of 20 control, saline-treated mice. Once a drug was demonstrated as being effective the test dose was progressively diminished by 50 per cent until one or more doses failed to significantly reduce mortality. Doses employed were usually in the range of 50, 25, 12.5, 6.0, 3.0 and 1.5 mgm./kgm., orally.

Increased mortality values occurred with decrements in dosage and were plotted on log-dose-probit charts (4) from which an estimation was made of the effective dose of each drug and its standard error (table 1). The effective dose represents the prophylactic dose which protected 50 per cent of animals which otherwise would have died following injection of epinephrine, i.e., the estimated dose which reduced mortality from 67.0 ± 1.53 per cent (controls) to 33.5 per cent. Analysis of the mortality data obtained from 40 groups of 20 control mice by application of the Chi Square test for homogeneity (5) indicated that the groups of mice were samples from a homogeneous population ($P = 0.50$).

The data presented are based upon the mortality percentages obtained 1 hour following injection of epinephrine. Observation of the animals was also made 18 hours after injection of epinephrine, but with none of the drugs was there an occurrence of late deaths or delayed symptoms which could be interpreted as indicating drug toxicity.

The selection of a two hour interval between oral drug treatment and injection of epinephrine was based on several known or demonstrated facts. In addition to the time required for absorption of the test drugs, it was necessary to allow for the fact that representative drugs from several chemical series under investigation were known not to exert maximal activity in reversing the pressor response to epinephrine and in blocking adrenergic nervous influence for 10 to 30 minutes, or longer, even after intravenous injection in dogs. Experiments were conducted with several adrenergic blocking drugs to provide important information concerning the onset and duration of action in mice. The data obtained (cf. figure 1 and comments under results) indicated that from the standpoint of convenience and

¹ This investigation was supported by a grant from Parke, Davis and Company, Detroit, Michigan.

reliability in routine screening of compounds an interval of 2 hours between drug treatment and injection of epinephrine would prove most useful in detecting activity of compounds irrespective of whether the onset of action was moderately early or late. Even a compound like Dibenamine (N,N-dibenzyl- β -chloroethylamine-HCl) (6, 7), with slow onset of action, exhibited activity 2 hours after administration provided the dose was sufficiently large (50.0 mgm./kgm. or more). It was assumed that selection of the 2 hour interval after oral administration would also provide for detection of compounds which were short-acting.

Acute toxicity following oral administration of three or four doses of each drug to groups of 20 mice (19-21 grams; non-fasted; both sexes) was estimated after plotting mortality percentages on log-dose-probit charts (4).

RESULTS AND COMMENTS

A. *Drugs which exert sympatholytic or adrenergic blocking effects.*

1. Yohimbine: This drug is known to block and reverse the pressor effects of epinephrine in cats and dogs and to block certain excitatory effects elicited by stimulation of adrenergic nerves. In mice (table 1), the effective dose of yohimbine was estimated as 23.0 ± 3.8 mgm./kgm., which represents nearly 25 per cent of the LD_{50} .

2. Prisol (2-benzyl-4,5,-imidazoline hydrochloride): This adrenergic blocking drug with an effective dose of 7.4 was three times as effective as yohimbine. This effectiveness of Prisol could possibly be related, in part, to a direct vasodilator action which has been demonstrated (8, 9, 10), especially since Mecholyl (vide infra) was found to decrease the toxicity of epinephrine in mice.

3. Dioxane derivatives: 2-Diethylaminoethyl-1,4-benzodioxane-HCl (883 F) and 2-piperidinomethyl-1,4-benzodioxane-HCl (933 F) are known to reverse the pressor responses to epinephrine and to diminish and block several responses which customarily follow adrenergic nerve stimulation. Furthermore, Bovet and Fourneau (11) have presented a meager amount of data which suggest that 883 F reduces the toxicity of epinephrine in mice, whereas it was stated that yohimbine failed to do so. To our knowledge these data are the only evidence that adrenergic blocking agents are capable of reducing the toxicity of epinephrine in mice, although an abstract of a report by Tani (12) claims that ergotamine, yohimbine, quinine, cinchonine, insulin and camphor are effective.

As indicated in table 1, both 883 F and 933 F, in oral doses of 25 and 50 mgm./kgm., were non-effective in reducing the toxicity of epinephrine injected 2 hours later in mice. The large doses of 50 mgm. also failed to confer protection when administered one hour before the toxic dose of epinephrine. Furthermore, 933 F failed to protect the mice when a dose of 25.0 mgm./kgm. was injected subcutaneously one hour before injection of epinephrine. Under these conditions, 883 F did significantly reduce mortality. The difficulty, or failure, in demonstrating that these compounds are capable of diminishing the toxicity of epinephrine in mice could scarcely be due to the toxicity of the doses employed since comparable doses of yohimbine (25.0 mgm./kgm.) were effective even though oral doses of yohimbine appear more toxic (table 1) than parenteral doses of 883 F and 933 F (11, 17). It is highly improbable that the oral doses of 883 F and 933 F (25 and 50 mgm.) were poorly absorbed since comparable oral doses in the dog have been reported to reverse the pressor response to epinephrine (13).

TABLE 1
Reduction of epinephrine toxicity by adrenergic blocking drugs

DRUG	DOSE ORALLY	MORTALITY	EFFECTIVE* DOSE \pm S.E.	ACUTE TOXICITY ORAL, MICE	LD ₅₀ /ED
	mgm./kgm.	per cent	mgm./kgm.	mgm./kgm.	
Saline-treated Controls		67.0†			
Yohimbine-HCl	25.0 12.5	30 60	23.0 \pm 3.8	86.7 \pm 9.6	3.7
Priscol	25.0 12.5 6.0	0 15 35	7.4 \pm 1.2		
883 F	50.0‡ 25.0 25.0§	60 80 35	Ineffective Ineffective 25.0	500 s.c. (ref. 11)	
933 F	50.0‡ 25.0 25.0§	60 60 65	Ineffective Ineffective Ineffective	180 i.p. (ref. 19)	
Dibenamine	75.0 50.0 25.0	10 40 70	48.0 \pm 4.9	>2000	>40
N-Benzohydryl-N-ethyl- β -chloroethylamine-HCl	25.0 12.5 6.0	0 15 40	7.5 \pm 1.1	977 \pm 63	130
N-Ethyl-N-(1-naphthylmethyl)- β -chloroethylamine-HCl	12.5 6.0 3.0	0 45 60	5.7 \pm 0.6	725 \pm 57	127
N-[β -(2-Biphenyloxy)-ethyl]-N-ethyl- β -chloroethylamine-HCl	12.5 6.0 3.0	0 35 60	5.5 \pm 0.6	759 \pm 53	122
Mecholyl chloride	100.0** 6.0†† 3.0†† 1.0††	35 15 35 55	Ca. 100 2.4 \pm 0.4	1100††	11

* The effective dose is an estimate of the prophylactic dose required to protect 50 per cent of mice which otherwise would have died following injection of epinephrine (see text).

† Mortality in 40 groups of 20 control mice equalled 67.0 ± 1.53 per cent; each of the other values are based on a single group of 20 mice.

‡ Not effective when administered orally either 1 or 2 hours before injection of epinephrine.

§ Injected subcutaneously 30 minutes before injection of epinephrine.

** Orally, 30 minutes before injection of epinephrine.

†† Injected subcutaneously 15 minutes before injection of epinephrine.

‡‡ From Molitor, H.: This Journal, 53: 337, 1936.

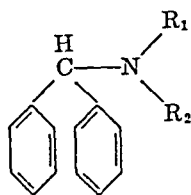
4. β -Chloroethylamines: The main purpose of developing a rapid screening method for detecting compounds possessing adrenergic blocking activity was the availability of several series of β -chloroethylamines chemically related to *N,N*-dibenzyl- β -chloroethylamine·HCl (Dibenamine), a compound which Nickerson and Goodman first reported (6, 7) as exerting adrenergic blocking action in several species.

When doses of 50 and 75 mgm./kgm. of Dibenamine hydrochloride were administered orally to mice the toxicity of epinephrine injected 2 hours later was diminished. Although protective activity was readily demonstrable under these conditions the magnitude of the effective dose (48 mgm./kgm.) provides no basis for the designation of Dibenamine as a potent adrenergic blocking agent (6, 7, 14) but is more in accord with the fact that comparatively large intravenous doses are required in cats and dogs to block adrenergic nerve stimulation and reverse the pressor response to epinephrine after a latent period of 30 minutes or longer. Following oral administration in mice the maximal effectiveness of Dibenamine was demonstrable after more than 2 hours (*vide infra*).

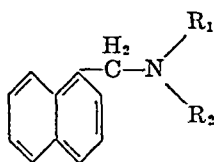
The synthesis of other β -chloroethylamines² provided a large number of compounds which proved effective in antagonizing the toxic effects of epinephrine in mice. Data presented in table 1 are typical of those obtained with several *N*-alkyl homologues in each of three series of compounds, the structure of which are as shown:

$R_1 = \text{alkyl } (\text{CH}_3\text{---C}_6\text{H}_{13})$

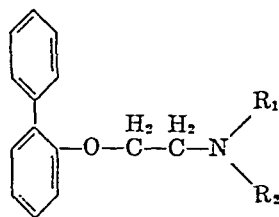
$R_2 = \beta\text{-chloroethyl } (\text{CH}_2\text{CH}_2\text{Cl})$



Benzohydrylamine



1-Naphthylmethylamine



β -(2-Biphenyloxy)-ethylamine

These compounds³ were remarkably effective in antagonizing epinephrine as revealed by the small magnitude of the oral effective dose (5.7 to 7.5 mgm./kgm.). Furthermore, as with Priscol, doses of 12.5 or 25 mgm./kgm. conferred full protection against the lethal action of epinephrine. The activity demonstrated could not be due to a strong vasodilator response, such as is produced with Mecholyl, since these β -chloroethylamines induce only a moderate depression of blood pressure in dogs which can be ascribed to blocking of adrenergic nerves.

² Synthesized and supplied to us by Drs. G. Rieveschl, Jr., R. Fleming and W. R. Coleman of Parke, Davis and Company Research Laboratories, Detroit, Michigan.

³ When compared on a molecular basis, *N*-ethyl-*N*-(1-naphthylmethyl)- β -bromoethylamine hydrobromide proved to be as effective as *N*-ethyl-*N*-(1-naphthylmethyl)- β -chloroethylamine hydrochloride.

In dogs, the drugs in intravenous doses of 1.0 to 5.0 mgm./kgm., were effective within 10 minutes as revealed by depressor responses to epinephrine and diminution, blocking or reversal of the pressor responses which otherwise follow splanchnic nerve stimulation, anoxia, carotid clamping and injection of nicotine (15, 16). The biphenyloxy and naphthylmethyl derivatives are not specific adrenergic blocking drugs since they antagonize some effects of histamine (2, 3) but this action could not account for protection against epinephrine in mice since the antihistamine drugs, Benadryl and Thephorin conferred no protection (table 2). Furthermore, neither Dibenamine nor N-benzohydryl-N-ethyl- β -chloroethylamine antagonize histamine and the evidence now available indicates that these drugs are specific adrenergic blocking drugs.

B. Specificity. In order to learn more concerning the specificity of the method for detecting adrenergic blocking activity several types of drugs other than adrenergic blocking agents were tested in mice. These drugs may be segregated into two classes:

1. Miscellaneous drugs not known to exert adrenergic blocking action.

- a. Vasodilator drugs: Acetyl- β -methylcholine chloride (Mecholyl), sodium nitrite, papaverine hydrochloride and aminophylline.

As revealed in table 1, a large oral dose of Mecholyl, or small doses injected subcutaneously, caused a significant reduction in mortality of epinephrine-treated mice. The well-known cardio-inhibitory and vasodilator effects of Mecholyl could account for the decreased toxic action of epinephrine. If reduction of epinephrine toxicity was due to the vasodilator action of Mecholyl it is at once apparent that strong vasodilator action must be exerted since other drugs such as sodium nitrite, papaverine, aminophylline and choline, which are less potent vasodilator drugs, were ineffective in reducing the toxicity of epinephrine (table 2). Raab and Humphreys (17) have recently reported that nitroglycerine diminished or abolished epinephrine-induced cardiac acceleration in cats although it failed to reduce the toxicity of epinephrine in rats.

Papaverine, in the oral dose of 25 mgm./kgm. failed to reduce the toxicity of epinephrine in mice despite its vasodilator action and its ability to decrease the irritability of the heart (cf. 17 for refs.). In rats, intraperitoneal injections of papaverine prolonged survival but did not significantly reduce mortality following injection of fatal doses of epinephrine (17). Papaverine injected intravenously in rabbits was reported to prevent epinephrine-induced pulmonary edema (18).

- b. Pentobarbital sodium: Mice receiving lethal or near lethal doses of epinephrine exhibited locomotor ataxia and a few clonic movements concurrent with a depressed state which could conceivably be exaggerated by a depressant drug such as pentobarbital sodium. However, even large doses of this barbiturate failed to significantly increase or decrease the toxicity of epinephrine. These results minimize the possibility that drugs with moderate depressant action would alter the toxicity of epinephrine. However, Luisada (18) found that narcotics and barbiturates diminished pulmonary edema and mortality rates of rabbits injected with epinephrine.

c. Quinine hydrochloride, quinidine sulfate, procaine hydrochloride and trasantin hydrochloride: In large doses, quinine and quinidine decrease the pressor response to epinephrine in dogs and decrease the irritability of the myocardium. Also, Tani (12) listed quinine as one of the drugs which decreased the toxicity of epinephrine in mice. For these reasons quinine and quinidine were tested to determine whether decreased epinephrine toxicity could be demonstrated. Procaine hydrochloride and trasantin hydrochloride were also tested since they exert a quinidine-like action on isolated auricular tissue of rabbits (19).

TABLE 2
*Drugs which failed to reduce toxicity of epinephrine in mice**

DRUG	DOSE, ORALLY, 2 HRS. BEFORE INJECTION OF EPINEPHRINE
	mgm./kgm.
Sodium nitrite.....	12.5, 25, 50
Papaverine hydrochloride.....	25
Aminophylline.....	50, 100
Choline chloride.....	100
Pentobarbital sodium.....	25, 50, 100
Quinine hydrochloride.....	50
Quinidine sulfate.....	6, 25, 50
Procaine hydrochloride.....	50
Trasentine hydrochloride.....	50
Ascorbic Acid.....	50
Nicotine.....	1, 6
Tetramethylammonium bromide†.....	3, 6
Tetraethylammonium chloride†.....	12.5, 25
Cocaine hydrochloride.....	12.5, 25
Atropine sulfate.....	1, 12.5
Benadryl hydrochloride.....	50
Thephorin tartrate.....	50

* With each of the drugs listed the indicated dose, when administered to a group of 20 mice, failed to significantly increase or decrease the mortality from that which occurred in a concurrent control group of 20 saline-treated mice.

† Administered intraperitoneally 30 minutes before injection of epinephrine.

Negative results obtained with these compounds render it less likely that a quinidine-like action accounts for reduced epinephrine toxicity in mice pretreated with adrenergic blocking drugs such as N,N-dibenzyl- β -chloroethylamine·HCl (Dibenamine) and several other chloroethylamine derivatives which bear some chemical similarity to N-methyl-dibenzylamine and derivatives, including α -fagarine, which exert strong quinidine-like action (20). Furthermore, the failure of 933 F to yield positive results indicates that its quinidine-like action (19) did not influence epinephrine toxicity.

2. Drugs which might enhance the toxicity of epinephrine.

a. Ascorbic acid: A comparatively large dose of ascorbic acid, a reducing agent which could possibly retard the oxidation of epinephrine and thereby enhance or prolong its action, failed to significantly increase the toxicity of epinephrine.

b. Ganglionic stimulating drugs: The toxicity of epinephrine was not increased by a non-toxic, oral dose of nicotine (1.0 mgm./kgm.) whereas a larger dose induced symptoms of nicotine intoxication and significantly increased mortality of epinephrine-treated mice. The ganglionic stimulating drug, tetramethylammonium bromide (21), in doses of 3.0 and 6.0 mgm./kgm., administered intraperitoneally 30 minutes before injection of epinephrine, failed to increase mortality.

Some increase of epinephrine toxicity was suggested by the mortality following intraperitoneal injection of the ganglionic blocking drug, tetraethylammonium chloride (22), but the increase in mortality following each of the doses (12.5 and 25 mgm./kgm.) was not significant.

c. Cocaine hydrochloride and ephedrine hydrochloride: The alleged ability of cocaine to enhance excitatory effects of epinephrine was not apparent in the form of increased toxicity of epinephrine in mice pretreated with the doses of cocaine used. Ephedrine, in the doses employed, also failed to increase epinephrine toxicity.

d. Atropine sulfate: After blocking the action of the parasympathetic nervous system with atropine, epinephrine did not prove more toxic in mice.

e. Antihistamine drugs: Most antihistamine drugs, including Benadryl, are capable of increasing the magnitude and duration of the pressor response to small doses of epinephrine in anesthetized dogs (cf. 23 for refs.). In contrast, under the same conditions, Thephorin decreases the pressor response to epinephrine (24), a fact which we have confirmed (15). Following oral administration of appreciable doses in mice, both Benadryl and Thephorin failed to alter the toxicity of epinephrine a significant degree.

C. *Onset and duration of action.* Various times (1, 2, 3, 4, 8 and 18 hours) were permitted to elapse following treatment with drugs or saline before injections of epinephrine were made. A group of 20 mice was used with each of the drugs at each time interval and a similar group of saline-treated mice served as concurrent controls (fig. 1).

Over the period of 18 hours the injection of epinephrine in groups of saline-control mice elicited mortality rates of 60 to 75 per cent. Dibenamine, in a dose of 25.0 mgm./kgm., failed to decrease the toxicity of epinephrine until the third hour; almost complete protection was conferred at the fourth hour, whereas epinephrine again exerted full toxic effects 8 and 18 hours after treatment with Dibenamine. This slow onset of action of Dibenamine in mice is in general agreement with the findings of Nickerson and Goodman (6, 7) who reported a delayed but prolonged action following intravenous injection in cats and dogs. In other experiments we have shown that larger, oral doses of Dibenamine in mice decreased the toxicity of epinephrine injected at 2 and even 24 hours later.

When directly compared with Dibenamine, a smaller dose (12.5 mgm./kgm.) of N-benzohydryl-N-ethyl- β -chloroethylamine hydrochloride exerted an early, pronounced and prolonged effect in reducing the toxicity of epinephrine (figure 1). Similar activity was exerted by N-ethyl-N-(1-naphthylmethyl)- β -chloroethylamine hydrochloride (12.5 mgm./kgm.) and the N-butyl derivative of N-

[β -(2-biphenyloxy)-ethyl]- β -chloroethylamine hydrochloride (6.0 mgm./kgm.). In fact, the data presented are typical of those obtained with several alkyl homologues in each of the three series of new synthetic compounds.

DISCUSSION. Oral administration of adrenergic blocking drugs, yohimbine, Priscol and Dibenamine, reduced the toxicity of epinephrine injected intraperitoneally in mice. The method, as described, thus provided a means of selecting active adrenergic blocking agents from three series of β -chloroethylamines. The data obtained with a representative of each series indicate that N-benzohydryl-N-ethyl- β -chloroethylamine, N-ethyl-N-(1-naphthylmethyl)- β -chloroethylamine and N-[β -(2-biphenyloxy)-ethyl]-N-ethyl- β -chloroethylamine, all as hydrochlorides, were markedly effective in reducing the toxicity of epinephrine. Addi-

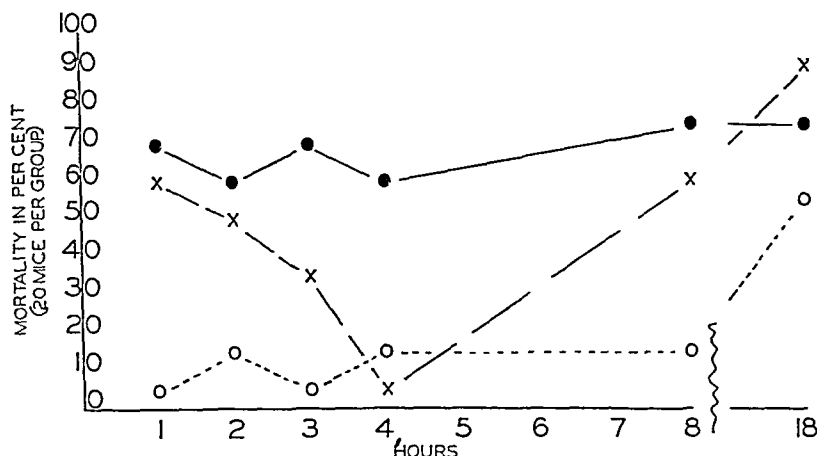


FIG. 1. REDUCTION OF EPINEPHRINE TOXICITY AT VARIOUS TIME INTERVALS AFTER TREATMENT WITH ADRENERGIC BLOCKING AGENTS

- — ● Concurrent controls (saline).
- x — x Dibenamine.HCl, 25 mgm./kgm.
- — ○ N-Benzohydryl-N-ethyl- β -chloroethylamine.HCl, 12.5 mgm./kgm.

tional, unpublished data reveal that substitution of higher alkyl groups for the ethyl group in these compounds seldom reduced the potency more than one-half or one-third provided that the compounds were readily soluble in water. Each of the β -chloroethylamines which reduced toxicity of epinephrine in mice, when injected intravenously in anesthetized dogs was found to cause epinephrine to lower the blood pressure. Furthermore, the β -chloroethylamines herein discussed also diminished, blocked or reversed the pressor responses which customarily follow enhanced sympathetic nervous activity induced by anoxia, carotid clamping, nicotine injections and faradization of splanchnic nerves (15, 16). Reduction of epinephrine toxicity in mice has proved a useful means of detecting compounds with adrenergic blocking action.

The data presented provide information concerning the reliability and speci-

ficity of the mouse screening method. Oral administration of comparatively large doses of the dioxane derivatives, 883 F and 933 F, failed to reduce epinephrine toxicity in mice, thereby indicating that one could not rely on the method for detecting this type of adrenergic blocking agent. Possibly the dioxane derivatives differ qualitatively from other adrenergic blocking drugs in their anti-epinephrine effects. However, Raab and Humphreys (14) have recently reported that 933 F, in a dose of 10 mgm./kgm., intraperitoneally, reduced the toxicity of epinephrine in rats.⁴

As regards specificity, it was demonstrated that Mecholyl chloride, in large oral doses and small subcutaneous doses, was capable of reducing epinephrine toxicity. Assuming that this effect of Mecholyl was due to a strong vasodilator action, it is pertinent to note that less potent vasodilator drugs (papaverine, sodium nitrite and aminophyllin) were not effective in the doses employed, and that nitroglycerin was non-effective in rats (18). Many other types of drugs (cf. table 2) proved non-effective. It can be concluded that the method is not entirely specific for detection of adrenergic blocking activity. Obviously, the adrenergic blocking action of active drugs selected by the mouse test should be verified by demonstrating such action in other animal species. If unable to verify such activity, it would be necessary to search for some pharmacological property which would explain the effects obtained in mice.

SUMMARY

The ability of chemical compounds to exert adrenergic blocking (sympatholytic) activity was usually detectable following oral administration of the compounds to mice since they became resistant to the lethal effects of epinephrine injected intraperitoneally. Reduction of epinephrine toxicity in mice was demonstrated with adrenergic blocking drugs such as yohimbine and Priscol, but not with the dioxane derivative, 933 F, although the closely related compound, 883 F, proved effective following subcutaneous administration. Effectiveness was demonstrated with Dibenamine (N,N-dibenzyl- β -chloroethylamine). The most effective compounds were representatives of three chemical series of new β -chloroethylamines including the hydrochlorides of N-benzohydryl-N-ethyl- β -chloroethylamine, N-ethyl-N-(1-naphthylmethyl)- β -chloroethylamine and N-[β -(2-biphenyloxy)-ethyl]-N-ethyl- β -chloroethylamine. These compounds also induced "epinephrine reversal" in cats and dogs and in the latter animal they diminished, blocked or reversed the pressor responses which follow increased sympathetic nervous activity induced by a variety of means.

The reduction of epinephrine toxicity in mice with a given compound does not necessarily indicate adrenergic blocking activity since the parasympathomimetic drug, Mecholyl, also reduced epinephrine toxicity.

⁴ In rats, Rothlin has recently demonstrated that natural and dihydrogenated ergot alkaloids reduce the toxicity of epinephrine injected intravenously (Bull. Acad. Suisse sc. med., 2: 1-24, 1946/47).

Acknowledgements. We are grateful to Dr. M. J. Schiffrin of Hoffman LaRoche, Nutley, New Jersey for the Thephorin tartrate; to Dr. M. Nickerson, Univ. of Utah School of Med., Salt Lake City, Utah, for a quantity of Dibenzamine; and to Dr. G. Rieveschl, Jr., of Parke, Davis and Co., Detroit, for Priscol, 883 F, 933 F, Benadryl, tetramethylammonium bromide and tetraethylammonium chloride.

REFERENCES

1. LOEW, E. R., AND MICETICH, A.: Fed. Proc., 6: 351, 1947.
2. LOEW, E. R., MICETICH, A., AND ACHENBACH, P.: Fed. Proc., 6: 351, 1947.
3. ACHENBACH, P., AND LOEW, E. R.: Fed. Proc., 6: 304, 1947.
4. MILLER, L. C., AND TAINTER, M. I.: Proc. Soc. Exper. Biol. and Med., 57: 261, 1944.
5. SNEDECOR, G. W.: *Statistical Methods*. Fourth Edition. The Collegiate Press, Inc., Ames, Iowa (1946).
6. NICKERSON, M., AND GOODMAN, L. S.: Fed. Proc., 5: 194, 1946.
7. NICKERSON, M., AND GOODMAN, L. S.: This Journal, 89: 167, 1947.
8. HARTMANN, M., AND ISLER, H.: Arch. f. Exper. Path. u. Pharmacol., 192: 141, 1939.
9. MEIER, R., AND MEYER, R.: Schweiz. med. Wschr., 71: 1206, 1941.
10. AHLQUIST, R. P., HUGGINS, R. A., AND WOODBURY, R. A.: This Journal, 89: 271, 1947.
11. FOURNEAU, E., AND BOVET, D.: Arch. Int. Pharm. et Therap., 46: 178, 1933.
12. TANI, S.: Fol. pharmacol. jap., 13: 28, 1932; (in Ber. ges. Phys. u. Pharm., 67: 199, 1932).
13. BING, R. J., AND THOMAS, C. B.: This Journal, 83: 21, 1945.
14. RAAB, W., AND HUMPHREYS, R. J.: This Journal, 88: 268, 1946.
15. Unpublished data.
16. STONE, C. A., ACHENBACH, P., AND LOEW, E. R.: Fed. Proc., 7: 258, 1948.
17. RAAB, W., AND HUMPHREYS, R. J.: This Journal, 89: 64, 1947.
18. LUISADA, A.: Arch. f. Exp. Path. u. Pharmacol., 132: 313, 1928.
19. DAWES, G. S.: Brit. J. Pharmacol. and Chemotherapy, 1: 90, 1946.
20. MOISSET DE ESPANES, E., AND WEKSLER, B.: Proc. Soc. Exper. Biol. and Med., 63: 195, 1946.
21. BURN, J. H., AND DALE, H. H.: This Journal, 6: 417, 1915.
22. ACHESON, G. H., AND MOE, G. K.: This Journal, 87: 220, 1946.
23. LOEW, E. R.: Physiol. Revs., 27: 542, 1947.
24. LEHMANN, G., HAGAN, E., BARBAROW, G., AND ROE, M.: Fed. Proc., 6: 350, 1947.

STUDIES ON NITRATE ESTERS

I. NITRITE-PRODUCING SYSTEMS IN RABBIT TISSUES

FRED W. OBERST¹ AND FRED H. SNYDER

Research Laboratories, The Wm. S. Merrell Co., Cincinnati, Ohio

Received for publication April 16, 1948

Various nitrate esters, such as glycerol trinitrate, erythritol tetranitrate, and mannitol hexanitrate, have for many years been used in the treatment of hypertension, but the exact mechanism of their action has not been well defined. Leech (1) believed that the nitrite ion found in the blood after ingestion of organic nitrates was responsible for the pharmacological response. The basic action of the nitrite ion in the body is the lowering of tone and the relaxation of arterial muscles, producing a prompt fall in blood pressure. Crandall et al. (2) observed that glycerol trinitrate disappears from the blood very rapidly following intravenous administration to dogs, and that nitrite could be detected qualitatively.

Hay (3) called attention to the similarity in action between glycerol trinitrate and sodium nitrite. He demonstrated that alkali converted about two-thirds of the nitrogen of glycerol trinitrate to inorganic nitrite, and that a faint trace of nitrite appears after the incubation of glycerol trinitrate with blood. The work of Herrman et al. (4) showed a striking parallelism between the rate of alkaline hydrolysis and the depressor action of a series of nitrate esters. Crandall (5) found that glycerol trinitrate disappears rather rapidly when incubated with fresh blood, and that nitrite could be detected by a qualitative test; the action was shown to be due entirely to the erythrocytes. Yagoda and von Oettingen (6) demonstrated the formation of inorganic nitrite from glycerol trinitrate, erythritol tetranitrate, and pentaerythritol tetranitrate on incubation with blood from dogs.

Krantz and his co-workers, on the basis of their studies, have taken the position that the action of nitrate esters is not related to reduction or nitrite formation, but is dependent on the intact molecule. They have shown (7) that, after administration of several nitrate esters, the amount of nitrite found in the blood is never sufficient to account for the observed depressor effect. They have also demonstrated (8) that the rate of alkaline hydrolysis may be completely unrelated to depressor action, since isomannide dinitrate, an effective depressor drug, is quite resistant to treatment with alkali. Further, they observed that, after the administration of amyl nitrite, there is a rapid disappearance of nitrite from the blood with the formation of nitrate; they suggest that there is a tendency toward oxidation rather than reduction in the nitrite-nitrate relationship. Confirmatory evidence has been supplied by Marshall (9), who stated that perfusion experiments with nitrate esters showed dilatation although no nitrite was present in the perfusate.

¹ Present address: The Murray Foundation, Inc., Cincinnati 29, Ohio.

It has long been known that biological fluids and cellular enzymes are capable of bringing about the reduction of inorganic nitrate to nitrite; this action has been demonstrated for milk (10, 11, 12), bacteria (13), and animal tissues (14). Aside from the previously cited studies on the formation of inorganic nitrite in the presence of blood (3, 5, 6), and a report of negative findings with frog muscle by Marshall (9), no reports have been found on the behavior of nitrate esters in the presence of biological materials *in vitro*.

The present study reports findings on the formation of nitrite ion from nitrate esters in the presence of blood and tissue suspensions. The nature of the system or systems responsible for this reductive breakdown has been investigated.

METHODS. Suspensions were prepared by homogenizing one part of fresh tissue with four parts of aqueous buffer solution in a Waring blender. Tissues were obtained from young white rabbits weighing about 2 kgm. Equal volumes of the homogenate and a solution of nitrate ester in the same buffer, each having been previously warmed to 37°C., were rapidly mixed with a high speed stirrer. Unless stated otherwise, the final ester concentration supplied an amount of nitrogen equivalent to 2.5 mgm. of nitrite ion (NO_2) per 100 cc. Samples for nitrite assay were removed immediately after mixing and at suitable intervals during 2 hours' incubation at 37°C. Nitrite concentrations were determined by the method of Yagoda and von Oettingen (6); N-naphthylethylene-diamine dihydrochloride, the coupling component described by Bratton and Marshall (15), was used for color development. Color intensities were measured in a Fisher photoelectric colorimeter with a No. 525 filter.

The compounds used were glycerol trinitrate, *l*-glucosan trinitrate,² and mannitol hexanitrate. Studies were also carried out on sodium nitrate, but there was no indication of the reduction of inorganic nitrate to nitrite ion under any of the experimental conditions employed. The values reported are based on at least three determinations made at different times with fresh tissue preparations.

RESULTS. *Fundamental Studies on the Nitrite-producing Systems.* Preliminary studies indicated that inorganic nitrite was formed rapidly when glycerol trinitrate was incubated with liver homogenate, but not with muscle homogenate or diluted whole blood. Since glycerol trinitrate was considered "typical" of the compounds being investigated, reactions in the presence of liver received primary attention. While appreciable variations in activity were observed among livers from different animals, these differences do not invalidate the general conclusions.

(a) *Effect of pH.* The rate of nitrite formation from glycerol trinitrate, *l*-glucosan trinitrate, and mannitol hexanitrate was measured with a series of buffer solutions of graded pH values. The reaction showed a fairly sharp pH maximum, with highest values observed in the neighborhood of pH 8.4 (fig. 1). The reaction in the case of mannitol hexanitrate appeared less sensitive to pH changes than that with the other two compounds. A borate buffer of pH 8.4 was used in all subsequent work. No difference was observed in the results with phosphate and borate buffers at the same pH value.

² The authors wish to express their appreciation to Mr. Lester P. Kuhn, of the Ballistic Research Laboratory, Aberdeen Proving Ground, Md., for supplying the sample of *l*-glucosan trinitrate used in these studies.

It was also demonstrated that the nitrite-forming system was, at least in part, irreversibly destroyed by acid. Homogenates acidified to pH 5 or below, and then re-neutralized, were less active in nitrite formation than were the original suspensions. However, a considerable part of the activity could be restored even after acidification to pH 2 (table 1).

(b) *Effect of Dilution.* Further dilution of the liver homogenate with buffer led to a considerable decrease in the rate of nitrite liberation from glycerol tri-

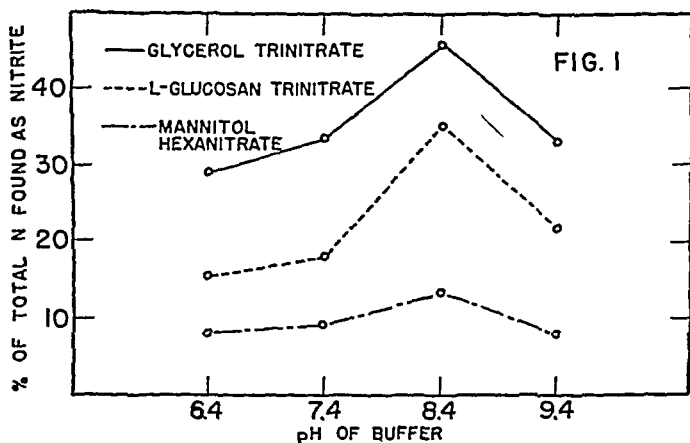


FIG. 1. LIBERATION OF INORGANIC NITRITE FROM NITRATE ESTERS INCUBATED FOR 2 HOURS AT 37°C. WITH RABBIT LIVER HOMOGENATES PREPARED WITH BUFFERS OF VARIOUS pH VALUES

TABLE 1

The effect of acidification and of re-neutralization on the production of inorganic nitrite from nitrate esters by rabbit liver homogenate

TREATMENT OF HOMOGENATE	PER CENT OF TOTAL N FOUND AS NITRITE		
	Glycerol trinitrate	<i>l</i> -Glucosan trinitrate	Mannitol hexanitrate
Buffered at pH 8.4	44	35	14
Acidified to pH 2.0	3	5	1
Acidified to pH 2.0, then adjusted to pH 8.4.	32	20	10

nitrate and *l*-glucosan trinitrate. However, the amount of nitrite formed from mannitol hexanitrate was not greatly diminished even when the original 10 per cent suspension was diluted ten-fold (fig. 2).

(c) *Effect of Heat.* Since inactivation by heat is a useful tool in the investigation of enzymatic reactions, studies were carried out to determine the effect of heat on the ability of liver to liberate nitrite ion from nitrate esters. In the case of glycerol trinitrate, it was found that a definite loss in activity occurred when

the liver homogenate was heated for 5 minutes at 55°C. before mixing with the ester; activity was not entirely lost, however, even after heating at 100°C. for 45 minutes. Mannitol hexanitrate behaved somewhat similarly, but the effect

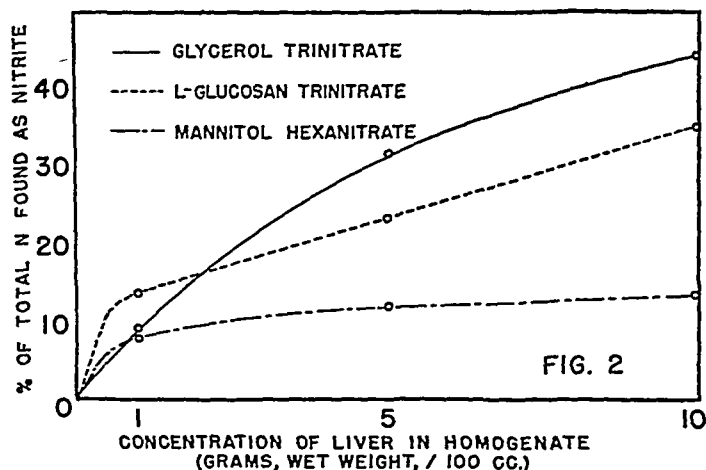


FIG. 2. NITRITE FORMATION FROM NITRATE ESTERS INCUBATED FOR 2 HOURS AT 37°C. WITH RABBIT LIVER HOMOGENATES OF SEVERAL CONCENTRATIONS

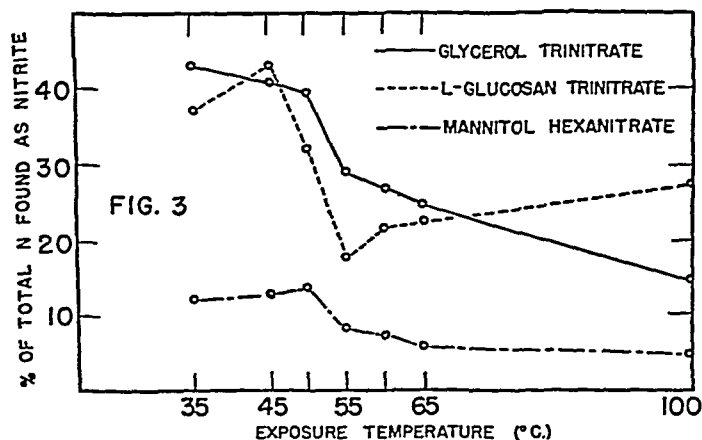


FIG. 3. NITRITE FORMATION FROM NITRATE ESTERS INCUBATED FOR 2 HOURS AT 37°C. WITH RABBIT LIVER HOMOGENATES PREVIOUSLY EXPOSED FOR 5 MINUTES TO THE TEMPERATURES INDICATED

on *l*-glucosan trinitrate was unique; partial inactivation took place at lower temperatures, and an increase in activity was observed in samples exposed to temperatures between 60° and 100°C. (fig. 3).

(d) *Effect of Ester Concentration.* Since many nitrate esters are only slightly soluble in aqueous buffer at pH 8.4, it seemed essential to demonstrate that differences observed among the compounds could not be entirely dependent on differences in solubility. Consequently, mannitol hexanitrate was studied at three different levels, equivalent to 1.25, 2.5, and 5.0 mgm. of nitrite per 100 cc. of final suspension. The amount of nitrite liberated increased with increasing ester concentration, indicating that saturation of the solution was not a limiting factor in nitrite formation (fig. 4). Estimation of initial reaction velocities by extrapolation of these data also indicated that there is at least rough conformity with Michaelis's theory (16).

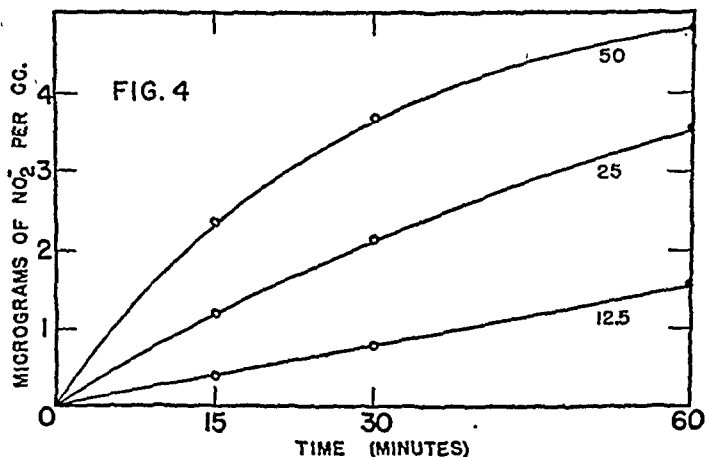


FIG. 4. THE RATE OF NITRITE FORMATION FROM MANNITOL HEXANITRATE, IN SEVERAL CONCENTRATIONS, INCUBATED WITH LIVER HOMOGENATES AT 37°C.

The figures on the curves indicate the amounts of mannitol hexanitrate added, in micrograms of nitrite (NO_2) per cc.

Comparative Studies on Three Esters. Table 2 summarizes the results with three compounds under several conditions. These data provide the basis for a study of some twenty additional nitrate esters, to be reported later.

(a) *Liver Homogenates.* The data show that glycerol trinitrate liberated a considerable amount of inorganic nitrite during 2 hours' incubation with liver homogenate, and that this value was greatly diminished when the tissue preparation was previously heated at 100°C. for 45 minutes. The effect of heat was much less pronounced in the case of *l*-glucosan trinitrate and mannitol hexanitrate.

(b) *Muscle Homogenates.* After it was demonstrated that the nitrite-forming action of liver on mannitol hexanitrate and *l*-glucosan trinitrate was incompletely destroyed by heat, the behavior of these compounds in the presence of muscle tissue was investigated. Only a very small amount of nitrite was liberated from

glycerol trinitrate in the presence of rabbit muscle homogenate, and this activity virtually disappeared in homogenates that had been heated in boiling water for 45 minutes. Somewhat more nitrite was found in the case of *l*-glucosan trinitrate, and considerably more with mannitol hexanitrate; in both instances, the rate of nitrite formation was increased by prior heating of the homogenate.

(c) *Blood*. Defibrinated blood, in a final dilution of 1:5 with buffer, liberated a very small amount of nitrite from glycerol trinitrate, a somewhat larger amount from *l*-glucosan trinitrate, and considerably more from mannitol hexanitrate. Heating the blood in boiling water for 45 minutes increased the amount of nitrite formed in all cases, and very greatly with *l*-glucosan trinitrate.

Discussion. It appears that at least two distinct systems are responsible for the formation of nitrite ion from nitrate esters under the conditions used. The first, present principally in liver, can be destroyed by heat. The second, occur-

TABLE 2
Production of inorganic nitrite from nitrate esters by rabbit tissues

TISSUE PREPARATION	PER CENT OF TOTAL N FOUND AS NITRITE		
	Glycerol trinitrate	<i>l</i> -Glucosan trinitrate	Mannitol hexanitrate
Fresh liver.....	44	35	14
Heated liver*.....	12	27	10
Fresh muscle.....	4	7	14
Heated muscle*.....	1	13	20
Fresh blood.....	4	8	16
Heated blood*.....	6	46	19

* Heated at 100°C. for 45 minutes, and adjusted to 37°C. before mixing with suspension of nitrate ester.

ring to at least some extent in liver, muscle, and blood, is not only heat-stable but is activated by heat, perhaps by liberation from a weakly combined form.

The reaction can not be explained in either case by simple hydrolysis of the esters followed by reduction of the nitrate ion to nitrite, since inorganic nitrate does not yield significant amounts of nitrite under identical conditions. The alternatives are (1) that the compounds are reduced to nitrite esters before hydrolysis, and (2) that the two reactions proceed together, mutually interdependent. In this connection, it would be interesting to study under the same conditions the fate of nitrous acid esters of polyhydric alcohols having different structural configurations.

SUMMARY

Inorganic nitrite is formed from nitrate esters of polyhydric alcohols by two systems occurring in normal rabbit tissues. The first, occurring principally in the liver, is heat-labile. The second, found in liver, muscle, and blood, is acti-

vated or released from combination by heat. The system as present in fresh liver homogenate shows maximal activity in the neighborhood of pH 8.4; after acidification to pH 2, it regains most of its activity on re-neutralization.

REFERENCES

1. LEECH, D. J.: *Brit. Med. J.*, 1: 1305, 1893.
2. CRANDALL, L. A., JR., LEAKE, C. D., LOEVENHART, A. S., AND MUEHLBERGER, C. W.: *This Journal*, 37: 283, 1929.
3. HAY, M.: *Practitioner*, 30: 422, 1883 (cited from Crandall (5)).
4. HERRMAN, R. F., LEAKE, C. D., LOEVENHART, A. S., AND MUEHLBERGER, C. W.: *This Journal*, 27: 259, 1926.
5. CRANDALL, L. A., JR.: *This Journal*, 48: 127, 1933.
6. YAGODA, H., AND VON OETTINGEN, W. F.: *Pub. Health Bull. No. 282*: 8, 1944.
7. RATH, M., AND KRANTZ, J. C., JR.: *This Journal*, 76: 33, 1942.
8. KRANTZ, J. C., JR., CARR, C. J., FORMAN, S. E., AND CONE, N.: *This Journal*, 70: 323, 1940.
9. MARSHALL, C. R.: *This Journal*, 83: 106, 1945.
10. BACH, A.: *Biochem. J.*, 33: 282, 1911.
11. HAAS, P., AND HILL, T. G.: *Biochem. J.*, 17: 671, 1923.
12. DIXON, M., AND THURLOW, S.: *Biochem. J.*, 18: 989, 1924.
13. FRED, E. B.: *Centr. Bakt. Parasitenk.*, 32: 421, 1912.
14. BERNHEIM, F., AND DIXON, M.: *Biochem. J.*, 22: 125, 1928.
15. BRATTON, A. C., AND MARSHALL, E. K., JR.: *J. Biol. Chem.*, 128: 537, 1939.
16. MICHAELIS, L., AND MENTEN, M. L.: *Biochem. Z.*, 49: 333, 1913.

p-AMINOMETHYLBENZENESULFONAMIDE (SULFAMYLON): ITS DETERMINATION IN BIOLOGICAL FLUIDS, WITH A PRELIMINARY REPORT ON THE BLOOD SERUM LEVELS OBTAINED UPON ORAL AND SUBCUTANEOUS ADMINISTRATION¹

M. LAWRENCE HEIDEMAN, JR., A.B.,² AND ROBERT C. RUTLEDGE, JR., M.D.³

Department of Pediatrics, Washington University School of Medicine, and the St. Louis Children's Hospital, St. Louis

Received for publication April 17, 1948

p-Aminomethylbenzenesulfonamide, known also as homosulfanilamide, 'Marfanil,' and 'Sulfamylon,'⁴ has evoked considerable interest in its properties and uses as a chemotherapeutic agent, especially in view of certain important differences from other sulfonamides. It is freely soluble in water; a solution containing 50 per cent by weight may be prepared (1). While there is considerable overlapping with other sulfonamides in its range of anti-bacterial activity, sulfamylon is especially effective against certain anaerobes, such as the gas gangrene group and other clostridia, and some gram-negative bacteria resistant to other sulfonamides. Sulfamylon was first used in Germany for the local treatment of gas gangrene and other wound infections (1). More recently it has been applied to the systemic therapy of urinary infections due to gram-negative bacilli, especially those not susceptible to other sulfonamides and to streptomycin (2). Other notable properties of sulfamylon are the definite bactericidal element in its effect and its continued action in the presence of para-aminobenzoic acid (3, 4). The lack of a method for the quantitative determination of sulfamylon has delayed the investigation of its pharmacology and clinical uses. Because sulfamylon is an aliphatic amine and is not diazotizable, the usual method (of Bratton and Marshall) for determining sulfonamides is not applicable to it.

After investigation of the optical properties of solutions of the drug, the absorption of radiation at 265 m μ was adopted as the basis of the spectrophotometric determination of sulfamylon in protein-free filtrates of biological fluids. Sulfamylon solutions show a marked absorption in the ultra-violet range of radiation with a well defined maximum at 265 m μ , and at this wave length optical density is proportional to concentration. The method devised is applicable to blood serum, whole blood, cerebrospinal fluid, pleural fluid, and ascitic and other edema fluids; it has not been applied to tissue extracts. It must be standardized separately for fluids differing markedly in protein content. Because of the presence of large amounts of interfering substances, which we have not succeeded in removing, application of this method to urine is not feasible.

¹ Much of this work was made possible by the assistance of the Children's Research Foundation.

² Junior Student, Washington University School of Medicine.

³ Junior Resident Physician, St. Louis Children's Hospital.

⁴ The material used in these studies was kindly furnished by the Winthrop Chemical Company, whose product is known as 'Sulfamylon.' For convenience the name sulfamylon will be used throughout this paper.

Reagents. 5 per cent $\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$ (aq.); 0.3 *N* $\text{Ba}(\text{OH})_2$ (aq.); diethyl ether (U.S.P. grade is satisfactory). The zinc sulfate is titrated with the barium hydroxide, using phenolphthalein as an indicator, and the appropriate dilution is made to effect equivalence.

Procedure. For routine studies a 1:10 dilution has been chosen for the filtrate. One ml. of the body fluid sample is added to 5 ml. of water, and to this dilution 2 ml. each of the adjusted zinc sulfate and barium hydroxide solutions are added to accomplish protein precipitation. After being shaken thoroughly and being allowed to stand for a brief period, the resulting suspension is centrifuged and the supernatant is decanted through a small filter. Four ml. of the filtrate and an equal volume of diethyl ether are placed in a glass-stoppered test tube and shaken on a mechanical shaker for 5 minutes; subsequent sharp separation of the layers is assured by centrifugation. The supernatant ether phase is drawn off and discarded, while the aqueous layer is placed in a fused silica absorption cell and the optical density read at 265 μ in the Beckman DU spectrophotometer against a water blank. A blank filtrate treated in exactly the same manner is also compared with water at 265 μ , since most biological fluids yield appreciable blanks. For estimation of high levels of sulfamylon, this blank may be prepared by the given procedure from any random normal sample of the body fluid concerned, but for maximal accuracy it should be prepared from a sample taken, previous to the administration of sulfamylon, from the same subject as the unknown.

The optical density obtained by subtracting the blank value from that of the unknown is converted to concentration of sulfamylon by multiplying by the appropriate constant determined by standardization. The method is standardized by adding a measured volume of known sulfamylon hydrochloride solution to a sample of the body fluid concerned and following the analytical procedure described above. Standardization at various concentrations of sulfamylon reveals results closely approximating a straight line when optical density is plotted against concentration.

EXPERIMENTAL

Solutions of sulfamylon show a sharp absorption peak at 265 μ . Practically no absorption of radiation is obtained in the near infra-red, visible, or long-ultra-violet regions of the spectrum, but a definite drop in transmission appears below 350 μ and is greatest at 265 μ , with transmission increasing rapidly again below this wave length. An aqueous solution of approximately 50 mg. sulfamylon hydrochloride per 100 ml. has the absorption characteristics shown in figure 1.

Measurement at 265 μ of the absorption of different aqueous solutions of pure crystalline, sulfamylon hydrochloride reveals that Beer's Law is followed closely (line A of fig. 2).

A comparison of filtrates of thirteen random blood sera, obtained from both adults and children of various ages and of both sexes, revealed some variation of optical density, which averaged 0.133 (73.5 per cent transmission) with an average deviation from the mean of 0.015. After extraction with an equal volume of ether the filtrates averaged 0.103 (79.0 per cent transmission) in optical density with an average deviation from the mean of 0.010. The absorption of radiation by blank filtrates depends somewhat on the degree of accuracy with which the Somogyi reagents are adjusted to each other. The series reported above utilized routine, coarsely-adjusted reagents, while with maximum accuracy of adjustment the optical densities of serum blanks generally fall within the range of 0.050 to 0.070. Although the average percentage variation of optical density of blank

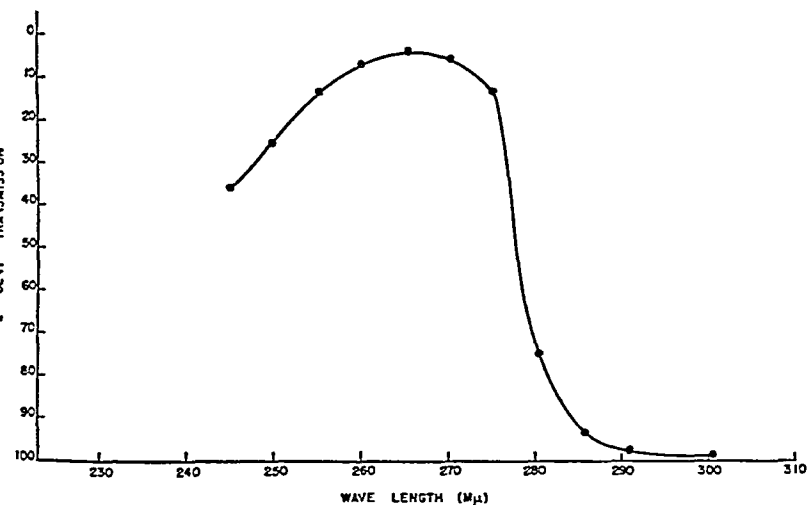


FIG. 1. ABSORPTION SPECTRUM OF SULFAMYLON HYDROCHLORIDE IN AQUEOUS SOLUTION 50 MG /100 ML.

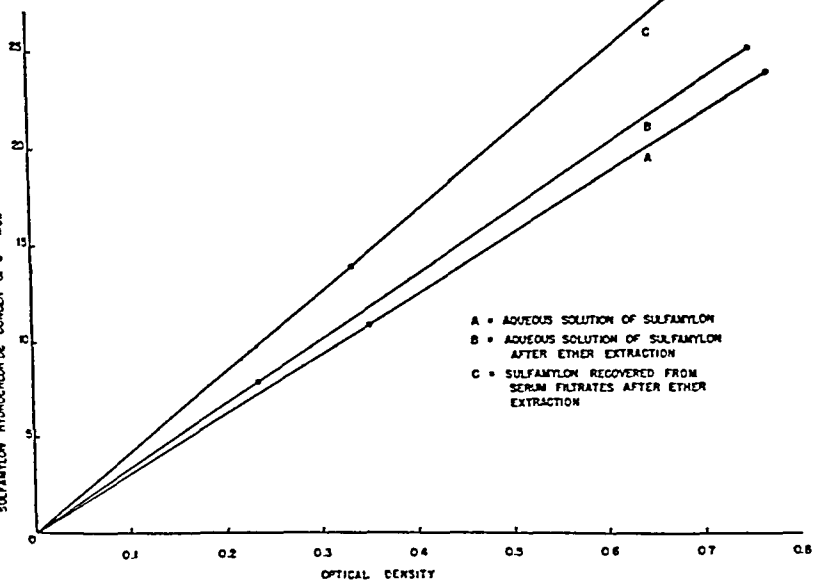


FIG. 2.

filtrates is almost as great after ether extraction as before, the absolute variation, which is the significant thing in this determination, is less after ether extraction.

Furthermore, great variations of single cases, which occasionally occur with random serum filtrates, are apparently prevented by the ether extraction. In addition, the optical density of filtrates after extraction remains constant for twelve to twenty-four hours, but without ether extraction changes may occur within a few hours. Hence ether extraction contributes to the accuracy and precision of the determination.

Strait, Aird, and Hrenoff (5) found an absorption peak at $265\text{ m}\mu$ in both normal and pathological specimens of cerebrospinal fluid to be due to ascorbic acid, while others have attributed it to nucleic acid. Irradiation with ultra-violet light was shown by them to obliterate this peak and to decrease the absorption at $265\text{ m}\mu$ to a low residual value. Because of these reports, filtrates of fresh

TABLE 1

PROCEDURE	concentration of sulfamylon $k = \frac{(\text{mg. } 100\text{ ml.})}{\text{optical densityat } 265\text{ m}\mu}$
1. Aqueous sulfamylon solution.....	$31.2 = \frac{25}{.801}$
2. Aqueous sulfamylon solution after ether extraction.....	$33.5 = \frac{25}{.746}$
3. Recovery from water after routine analytical procedure of adding precipitating reagents and extracting filtrate with ether.....	$37.5 = \frac{25}{.667}$
4. Recovery from normal spinal fluid.....	$38.7 = \frac{25}{.646}$
5. Recovery from ascitic fluid.....	$39.0 = \frac{25}{.641}$
6. Recovery from serum.....	$40.2 = \frac{25}{.622}$
7. Recovery from whole blood.....	$50.6 = \frac{25}{.494}$

For each of the above procedures the final concentration varied from 5 to 50 mg. per 100 ml. The "k" values were calculated from the mid-portion of each line and represent the slopes of the lines.

sera were exposed to ultra-violet radiation for 30 minutes and others were incubated at $37^{\circ}\text{C}.$ in open vessels for 30 minutes or longer in order to destroy ascorbic acid. No significant changes in absorption were produced by these procedures, nor was the optical density of a filtrate exposed to room temperature for 2 days decreased significantly. It seems quite possible that this residual absorption is primarily a property of aromatic compounds, especially aromatic amino acids, in the serum filtrate. This concept is suggested by their close structural similarity to sulfamylon and is supported by the finding that an aqueous solution of tyrosine has definite absorption of radiation in the ultra-violet, with a maximum at $275\text{ m}\mu$.

Additional studies were carried out in blood serum by adding 5 ml. of standard aqueous sulfamylon hydrochloride solution to 1 ml. of the serum and following

the analytical procedure described. Line C of figure 2 presents the data obtained from these studies, with optical density plotted against concentration of sulfamylon in the filtrate. Comparison of lines B and C reveals that in the analysis of serum a further loss of sulfamylon occurs, beyond that due to ether extraction. This loss is also proportional to concentration and must be attributed to co-pre-

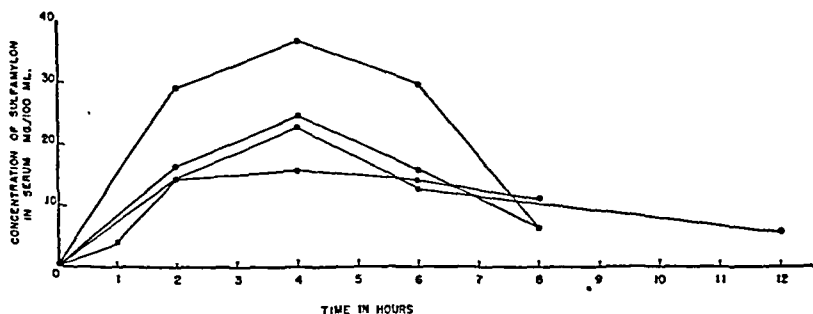


FIG. 3. SERUM LEVELS RESULTING FROM THE ORAL ADMINISTRATION OF SULFAMYLON 0.6 GM /KG.

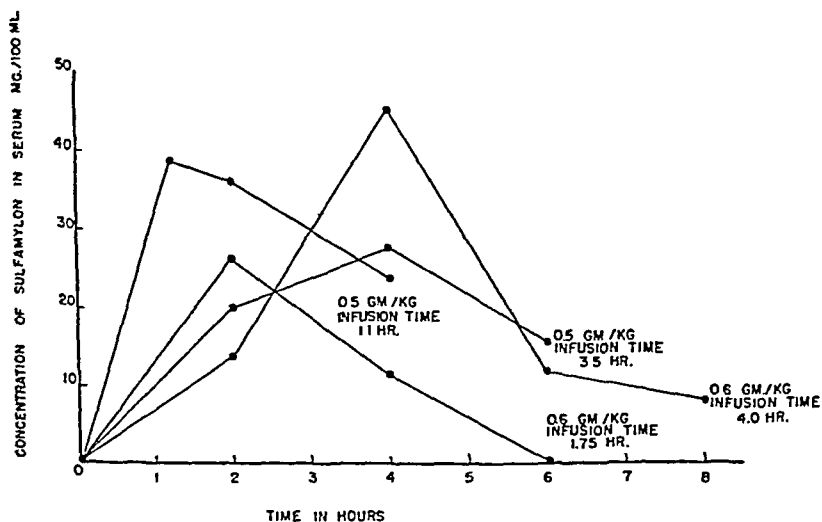


FIG. 4. SERUM LEVELS RESULTING FROM THE SUBCUTANEOUS ADMINISTRATION OF SULFAMYLON IN 1 PER CENT SOLUTION

cipitation of sulfamylon. Because of this phenomenon, the method must be standardized by addition of sulfamylon dilutions to the sample of biological fluid concerned before the proteins are precipitated, so as to reproduce the conditions of determinations of actual unknown levels. To clarify details of the co-precipitation further, addition studies were carried out on water, cerebrospinal fluid, ascitic fluid, blood serum, and whole blood. The results are shown in table 1.

It is evident from these data that co-precipitation occurs with both the barium sulfate (cf. examples 2 and 3 of table 1) and the proteins (cf. examples 3-7 of table 1); hence the method must be standardized separately for fluids differing considerably in protein content. However, the magnitude of any error caused by normal variations in plasma proteins would obviously be too small to be of significance.

Analysis of sera obtained from patients receiving penicillin, streptomycin, or salicylates demonstrated that these substances do not interfere with this spectrophotometric determination of sulfamylon. However, it is subject to marked interference due to other sulfonamides. As might be expected on the basis of their structural similarity to sulfamylon, they cause considerable absorption of radiation at 265 $m\mu$.

It is evident that the absolute accuracy of this method of analysis cannot be checked in the absence of a completely specific method of determining sulfamylon. However, precision has proved good and upon routine analyses of serum the error is well below 5 per cent, except with very low concentrations.

The method described has proved satisfactory in the study of the absorption of sulfamylon. Representative serum level curves obtained after the administration to convalescent infants and children of single oral and subcutaneous doses of sulfamylon are depicted in figures 3 and 4. The rapid excretion of the drug and comparatively high dosage to obtain the blood levels shown are evident. Full details and discussion of the findings upon oral and subcutaneous administration of sulfamylon will be given in a subsequent article.

SUMMARY AND CONCLUSIONS

A simple, rapid spectrophotometric method for the determination of p-aminomethylbenzenesulfonamide ('Sulfamylon,' homosulfanilamide, 'Marfanil') is described and the rationale of its procedure discussed.

This method is applicable to blood serum, blood, cerebrospinal fluid, pleural fluid, ascitic fluid, and other edema fluid, but not to urine because of the presence of large amounts of interfering substances. Although not specific, in view of the appreciable absorption of radiation at 265 $m\mu$ by other substances, it is believed to give good results under well-regulated conditions, as described in the procedure. Other sulfonamides are the only interfering substances likely to be present in significant amounts in body fluids to which this determination is applicable.

A preliminary report of the blood serum levels obtained after oral and subcutaneous administration of sulfamylon is presented, with graphic representation of the data.

Grateful acknowledgement is made to Dr. Alexis F. Hartmann for suggestions and assistance during the course of this work and in the preparation of this paper.

REFERENCES

1. Winthrop Chemical Company. Brochure, 1947.
2. Unpublished data, Dr. Alexis F. Hartmann.
3. GOETCHIUS, G. R., AND LAWRENCE, C. A.: *J. Bacteriology*, 49: 575, 1945.
4. WELCH, A. D.: *Physiological Reviews*, 25: 687, 1945.
5. STRAIT, L. A., AIRD, R. B., AND HRENOFF, M. K.: *Science*, 106: 64, 1947.

DYNAMICS OF RECOVERY AND MEASURE OF DRUG ANTAGONISM. INHIBITION OF SMOOTH MUSCLE BY LYSOCITHIN AND ANTIHISTAMINICS

M. ROCHA E SILVA AND WILSON T. BERALDO¹

Instituto Biológico,² São Paulo, Brazil

Received for publication April 17, 1948

The most common method for testing antagonism toward spasmogenic agents acting upon smooth muscle consists in adding a known amount of antagonist to the perfusing bath, where it is retained for a standard time, say one minute, before adding the test dose of the spasmogenic substance. As regards histamine and antihistaminics, this method was extensively used by Loew et al. (1). Various dilutions of each spasmogenic and antispasmodic drug were alternately employed in order to approach dilutions of the antagonist that would diminish contraction by 75 to 100 per cent. Essentially the same principle was involved in the method described by one of us (2) consisting in establishing the dose of antagonist (histidine, arginine derivatives, and histamine compounds) which would extinguish the spasmogenic action produced by a certain amount of histamine added to the bath, after a certain time following exposure to the antagonist. This method was also used by Morris and Dragstedt (3) to show the antihistamine effect of imidazol. A similar extinction method was employed by Mayer et al. (4) to screen the antihistamine activity of many synthetic substances, including pyribenzamine. The main characteristic of these methods consists in the fact that only one figure is used for the final calculation of the potency of the antagonist, and therefore a rather extensive *waste* of experimental data is inherent to all of them. More data can be obtained only through repetition of the same assay, some times very laborious, since recovery of the muscle takes a long time to be complete. The new method devised by Schild (5) in which "a new scale (pA_x) is defined as the negative logarithm to base 10 of the molar concentration of the antagonist which will reduce the effect of a multiple dose (x) of an active drug to that of a single dose," offers many advantages, but still involves the same principle mentioned above. The other characteristic of these methods consists in measuring the dose or concentration of the antagonist which enters into equilibrium with a certain dose or concentration of the active drug in their competition for the same receptors. Data obtained follow very closely the formula established by Gaddum (6) for the antagonism atropine-acetylcholine and adrenaline-ergotamine. Although derived from dynamic considerations, Gaddum's formula is valid once equilibrium is attained, establishing essentially static relationships between antagonist and active drug concentrations.

In the present paper, a simple method is outlined in which the speed of recovery of the muscle after the action of the antagonist is employed as a measure

¹ From the Department of Physiology, Faculty of Medicine, Univ. of São Paulo.

² Department of Biochemistry and Pharmacodynamics.

for drug antagonism, and the time of the assay is used entirely for getting data that will determine the final value of the antagonist potency. Since time of recovery is not influenced by repeated additions of the active drug, two or three spasmogenic agents can be assayed simultaneously upon the same piece of intestine, giving therefore highly comparable results, in the form of a "spectrum" of inhibition. The other advantage of the method consists in allowing a quantitative analysis of the important phenomenon of recovery of smooth muscle after inhibition, not only by the so-called specific antagonists, but also by nonspecific damaging agents, such as heat and cold, cell poisons, anesthetics, etc. Moreover, the use of the method described in this paper led to an analysis of the other phase of the competition problem, not covered by Gaddum's formula, namely dissociation of the hypothetical complex formed between antagonist and receptors, after washing out the remnants of the inhibitor from the outside bath. The results presented here fit more naturally with the idea that the antagonists studied disturb cell structure and that recovery of the muscle depends upon an auto-catalytic *regeneration* of the receptors.

EXPERIMENTAL

Preparation of lysocithin. Twenty one cc. of a $\frac{1}{2}$ dilution of egg yolk in saline, were incubated at 38°, in the presence of 4 cc. of a solution of cobra venom (*Naia naia*) containing 1 mg. per 1 cc. Incubation was prolonged for 12 or 14 hours, with a few drops of toluol as preservative. The active mixture was then poured into 250 cc. of hot ethyl alcohol and filtered while hot. The alcohol was evaporated to dryness *in vacuo* and the residue taken up in acetone, well shaken and collected by filtration. The material was further dried by several additions of fresh acetone and finally dissolved in saline for the assay. After standing in the ice-box, an inert precipitate is formed that can be removed without loss of potency of the clear and foamy supernatant. The venom of the indian cobra (*Naia naia*) was supplied through the courtesy of Dr. C. H. Kellaway, of the Wellcome Research Foundation, London.

Synthetic antihistamine agents. Neo-antergan in the form of the malonate of N-(p-methoxybenzyl)-N-dimethylaminoethyl, alpha-aminopyridine, was kindly supplied by Dr. R. Throwers, of the May and Baker Ltd., Dagenham Essex, England. Pyribenzamine (the hydrochloride of N-benzyl-N-dimethylaminoethyl, alpha-aminopyridine), antistine (2N-phenyl-N-benzylamino-methylimidazol) and trasentine (beta-dimethylaminoethyl-diphenylacetate hydrochloride) were supplied through the courtesy of Dr. R. L. Mayer, of the Ciba Pharmaceutical Products, Inc., Summit, N. J. Benadryl (beta-dimethylaminoethyl-benzhydryl ether hydrochloride) was supplied through the kindness of Dr. G. Rieveschl, of the Parke, Davis and Co., Detroit, Michigan.

Routine of the assay. Before starting the assay, the piece of guinea pig ileum is submitted to several additions of the same dose of histamine, in order to give a set of regular responses to the same dose of the active drug. The height of these regular contractions is taken as 100 per cent of response, to a dose of histamine giving moderate (not maximal) contraction. Since all calculations are carried out upon percentages, the actual amount of histamine added does not affect the results, provided the differences are not very large. Under the conditions of our experiments, the dose of histamine added was usually 0.2 to 0.4 cc. of 1:2 million solution of the base. The amount of the inhibitor to be tested was added to the perfusing bath containing the muscle and kept in contact with it for *exactly* one minute, the muscle was then washed with three changes of fresh Tyrode's Solution and the first dose of the stimulating agent (histamine, acetylcholine, KCl, etc.) was added exactly one minute after the removal of the inhibitor. The contraction of the muscle and changing of

perfusing fluid take 30 seconds; after that time, a resting period of one minute is allowed to elapse before the addition of the same dose of the spasmogenic agent. Thirty more seconds and one more minute elapse before the third addition and so on. The time of the first addition is taken as origin (0) and the first contraction, calculated as a percentage of the control contraction, is indicated by P_0 ; the successive contractions, similarly calculated as percentages of the controls, are indicated as P , at time t .

The assay was performed in a Dale's apparatus, with a chamber of 7 cc. capacity, after immersion of the piece of the guinea pig ileum (about one inch long). The latter was carefully opened at the attaching points in order to allow a free circulation of Tyrode's Solution through the lumen. The lever used was constructed according to the specifications of Schild (5) for an approximately linear and isotonic frontal writing. It was devised with the "purpose to ensure that the relation between shortening of the gut and effect on the drum should be linear." If the angle of the lever with the horizontal does not exceed 35° , that purpose is practically attained (7).

RESULTS

Lysocithin. When the venoms of certain species of snakes are incubated with egg yolk, they generate a powerful hemolytic substance—lysolecithin or lysocithin—by splitting off a molecule of oleic acid from lecithin (8, 9). This effect

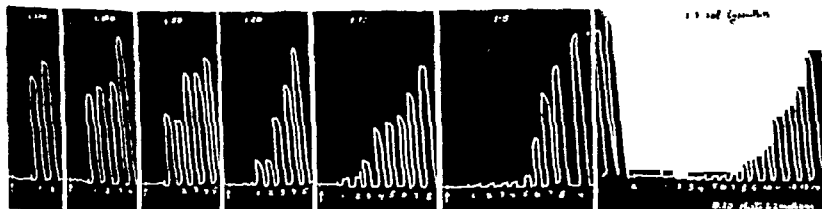


FIG. 1. LYSOCITHIN SOLUTION IN SALINE

In each panel, 0.2 cc. of different dilutions of a strong solution (1/1) of a purified lysocithin preparation. Testing solution: histamine 1:2 millions (0.2 cc.).

is due to the action of an enzyme—lecithinase—present in the venoms of many species of snakes (*Naia naia*, *Denisonia superba*, *Crotalus t. terrificus*, etc.). An extensive study of the pharmacological effects of lysocithin was presented by Houssay (10) and Feldberg and Kellaway (11). The latter authors described the effects of lysocithin upon the gut and showed that it reduces the excitability to several agents, e.g. histamine and acetylcholine, and also reduces the spontaneous activity of the intestine of several species of animals.

The inhibitory effect of a moderate dose of lysocithin is not instantaneous, requiring a certain time to be effective. If lysocithin is kept for one minute in contact with the gut and then washed out and recovery of the muscle tested at 1.5 minute intervals, a regular curve can be obtained that follows a simple mathematical equation that will be deduced in another section. If the dose is increased, a correspondingly increased effect, as measured by the time for 50 per cent recovery (R_{50}), is obtained (fig. 1). If one plots in relation to time the percentages of effects of the same dose of histamine, after washing out the lysocithin, taking the initial contraction as 100 per cent, the time necessary for 50 per cent

recovery (R_{50}) will measure the amount of lysocithin assayed. Using this annotation, the formation of lysocithin can be quantitatively timed. If now, the R_{50} for different doses are plotted in an arithmetic scale against the doses of lysocithin, a curve approaching a hyperbola, with an asymptote approaching 1100 seconds can be obtained (fig. 2 a). As for other kinds of antagonism, the most useful part of the curve is that comprised between 20 and 80 per cent of maximum effect, in this case, for values of R_{50} comprised between 200 and 800 seconds, approximately. In this range, the dose-effect curve approaches a straight line, especially when the dose of antagonist is plotted in a logarithm scale (fig. 2 b). These curves can be used to calculate, in arbitrary units, the amount of lysocithin present in a solution, if one takes, for instance as a unit, the amount of lysocithin which produces a 50 per cent recovery in one minute (time 0). Negative values for R_{50} indicate, of course, that recovery over the 50

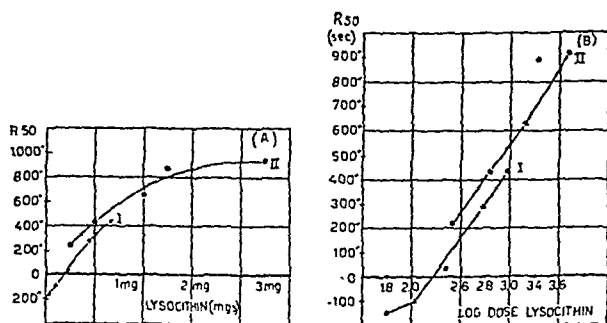


FIG. 2. THE SAME DRIED PREPARATION OF LYSOCITHIN ASSAYED UPON TWO DIFFERENT ILEUMS (I AND II)

(A) arithmetic scale; (B) the values of R_{50} are plotted against log. of dose of the lysocithin preparation.

per cent level took less than one minute, or that inhibition was not strong enough to cross the 50 per cent ordinate.

The curve correlating the amount of antagonist added to the intensity of effect as measured by the R_{50} index, is of the same kind as those obtained with other inhibitors such as atropine toward acetylcholine (for other examples, see Clark (12)) and benadryl toward histamine (13). The shape of the curves can be interpreted as indicating an adsorption phenomenon, as studied by Langmuir (14). In the case of very potent antihistamine agents, as pyribenzamine and neo-antergan, that show a tendency for irreversibility when relatively small doses are applied, this effect is not so clearly seen. It is, however, interesting that such a general law is again verified by using an entirely different method of measurement, as the one described above.

As shown in figure 3, the inhibitory effect of lysocithin depends considerably upon the time of contact with the muscle. It is not specific toward histamine and is even slightly stronger toward acetylcholine, as shown in figure 4, in which

the "spectrum" of inhibition has been obtained by using the method described above. A few additional facts should be mentioned here. First, the rate of recovery is independent of the number of additions of the spasmogenic agent.

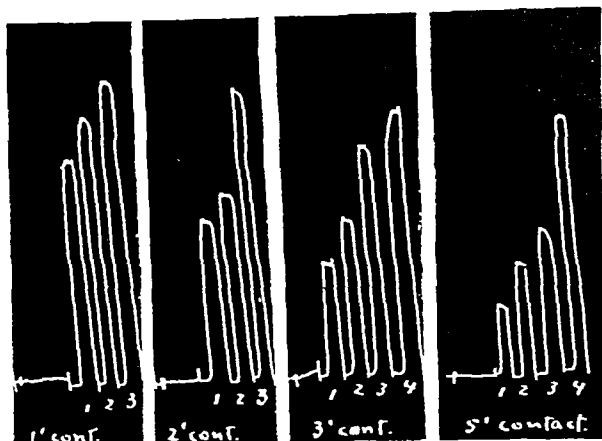


FIG. 3. EFFECT OF 120 γ OF A DRIED PREPARATION OF LYSOCITHIN, KEPT IN CONTACT WITH THE MUSCLE FOR INCREASING INTERVALS OF TIME

Testing dose: histamine 1:2 millions (0.2 cc.)

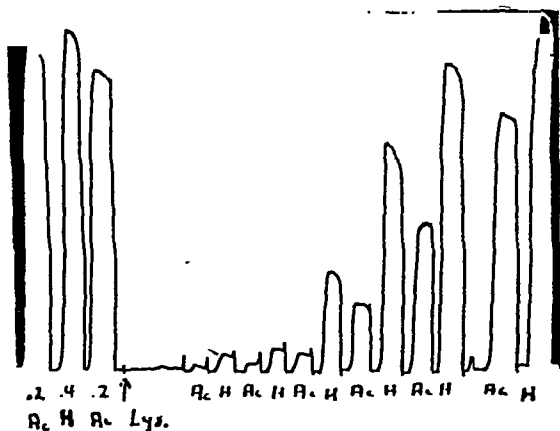


FIG. 4. SPECTRUM OF INHIBITION PRODUCED BY LYSOCITHIN

A dilution 1/10 of a strong lysocithin preparation was added at the arrow and washed out one minute later. Equipotent doses of acetylcholine and histamine were added alternately, every 1.5 minute. Recovery toward histamine was more rapid than toward acetylcholine.

Even large doses of histamine did not alter appreciably this rate of recovery. If recovery to lysocithin is allowed to proceed at two different temperatures, as 37° and 27°, the R_{50} is changed considerably from 506" at the higher temperature,

to 930" at the lower temperature, suggesting that a chemical phenomenon is involved in recovery.

Synthetic antihistamine drugs. Similar results were obtained by using benadryl, pyribenzamine, antistine and trasentine, as inhibitors. Neo-antergan, in the dose of 0.1 to 1.0 γ gave somewhat irreversible inhibition and therefore was less used than the other antihistamine substances. With benadryl, the same kind of irreversibility was obtained when the dose went up to 20 or 40 γ . Interesting "spectra" of inhibition were obtained by using alternately acetylcholine, histamine and KCl as spasmogenic agents in the assays. They have been omitted because they were only confirmatory of data already found in the literature (5, 15).

It is quite impressive that so different agents as lysocithin, benadryl, pyribenzamine, and also, as shown in a future paper, higher doses of acetylcholine and histamine, as well as octyl alcohol, lead to a kind of inhibition, the recovery from which follows essentially the same law. Such an analogy was rendered even more striking through calculation of the kinetics of recovery, as shown in the following section. All these facts constituted strong indication that recovery from inhibition depends solely upon the intimate potentialities of the muscle, and by no means from the dissociation of a complex supposedly formed between antagonist and cell receptors.

Kinetics of recovery. The shape of the curve correlating R_{50} to the dose of inhibitor, as well as the work generally known upon the mechanism of inhibition produced by antagonists as atropine, benadryl, etc., indicate that the primary effect of the drug is one depending on adsorption upon cell receptors. Of an entirely different nature appears to be the other part of the phenomenon, after the drugs have been washed out from the external bath. Since adsorption derives from the interaction of attractive forces not unrelated to those commanding chemical affinity (Langmuir (14)), one might assume that the drug combines with the cell receptors, forming a chemical complex that starts to dissociate as soon as the antagonist is washed out from the outside bath. This assumption is actually implied in the calculation of Gaddum's equation, since it is stated that "the rate of dissociation is proportional to the amount of drug fixed, or to the number of receptors occupied by the drug" (Clark (12)). Assuming that the percentage of contraction P_0 , at time 0, is a linear measure of the number of receptors left free by the antagonist, $100 - P_0$ will indicate the number of receptors blocked by the antagonist at the origin of time and therefore $a = 100 - P_0$ will measure the concentration of the hypothetical complex at the origin of time. Assuming that this complex will break up following the law of a monomolecular reaction, $x = P - P_0$ will indicate the decrements of (a) after a time (t), and the formula for constant k can be easily transformed into:

$$k = \frac{1}{t} \log \frac{100 - P_0}{100 - P} \quad (1)$$

As indicated in table 1, if constant k is calculated according to this equation, it continuously increases. If one plots (fig. 5) the different values of "constant"

TABLE 1

Examples of calculation of constant k' for lysocithin, benadryl, pyribenzamine and antistine

LYSOCITHIN (3 mcg.)				BENADRYL (5 γ)			
t (min.)	P (%)	k	k'	t (min.)	P (%)	k	k'
0 to 6.0	0	—	—	0 to 3.0	0	—	—
7.5	2.7	0.001	0.040	4.5	10.0	0.010	(0.10)
9.0	5.4	0.003	0.052	6.0	17.0	0.013	0.076
10.5	10.8	0.005	0.044	7.5	30.0	0.021	0.070
12.0	16.0	0.006	0.039	9.0	40.0	0.025	0.063
13.5	37.5	0.015	0.040	10.5	73.0	0.053	0.073
15.0	48.5	0.019	0.039	12.0	80.0	0.057	0.072
16.5	62.0	0.025	0.040	13.5	85.0	0.061	0.072
18.0	86.0	0.047	0.051	15.0	100.0	—	—
k' (average) \pm SE			0.044 \pm 0.002	k' (average) \pm SE			0.071 \pm 0.002
R ₁₀ (found) = 912" R ₁₀ (calc.) = 820"				R ₁₀ (found) = 588" R ₁₀ (calc.) = 510"			

PYRIBENZAMINE (0.2 γ)				ANTISTINE (10 γ)			
t (min.)	P (%)	k	k'	t (min.)	P (%)	k	k'
0	4.7	—	—	0	6.7	—	—
1.5	9.5	0.014	0.15	1.5	13.3	0.022	0.16
3.0	26.2	0.037	0.14	3.0	33.0	0.048	0.15
4.5	40.5	0.045	0.11	4.5	33.0	0.032	0.10
6.0	62.0	0.066	0.11	6.0	66.0	0.073	0.11
7.5	70.0	0.067	0.10	7.5	83.0	0.098	0.12
				9.0	100.0	—	—
k' (average) \pm SE			0.12 \pm 0.01	k' (average) \pm SE			0.13 \pm 0.01
R ₁₀ (found) = 300" R ₁₀ (calc.) = 301"				R ₁₀ (found) = 324" R ₁₀ (calc.) = 280"			

Note: R₁₀ (found) was determined directly upon the curves of recovery; R₁₀ (calc.) was calculated from equation III.

k against the values of P , a straight line is obtained, showing that variations of k follow the same law as that for the increase of percentages of effect (free receptors). If now, a new quantity is defined:

$$k' = \frac{100}{tP} \log \frac{100 - P_0}{100 - P} \quad (II)$$

the values obtained are fairly constant for the whole process of recovery. This simple and attractive correlation between k and P , strongly suggests that the liberated receptors, if the process is one of liberation, influence the rate of appear-

ance of new receptors, in such a way that the reaction follows an auto-catalytic course.

Table 1 and figure 7 show examples of calculation and fitting of experimental data and theoretical values deduced from equation II. This formula for k' gives a satisfactory description of the phenomenon in most cases and was calculated for a considerable number of experiments with different inhibitors (table 2). It is interesting that for all antagonists used in these experiments, when inhibition is measured by a certain value of R_{50} , the order of magnitude of k' is the same.

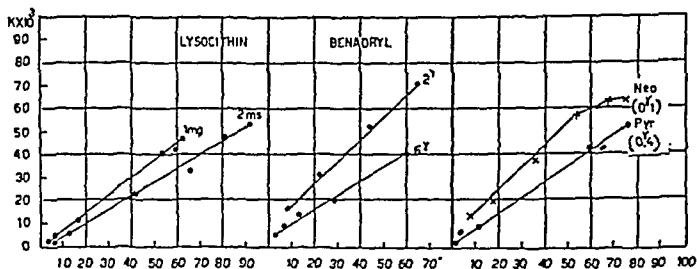


FIG. 5. CORRELATION BETWEEN THE VALUES OF FIRST ORDER "CONSTANT" k AND PERCENTAGE OF RECOVERY FROM INHIBITION BY LYSCITHIN, BENADRYL, NEO-ANTERGAN AND PYRIBENZAMINE

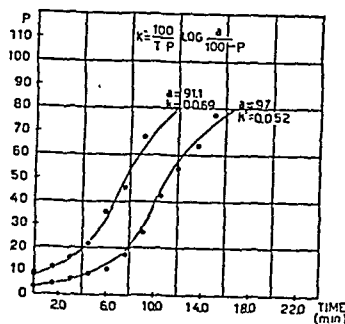


FIG. 6. RECOVERY FROM INHIBITION BY LYSCITHIN

The theoretical curves were calculated from equation II. In one curve, k' is exactly the average of experimental values. In the other curve, k' was chosen as 0.052 that is not significantly distinct from the experimental average (0.054). Points represent experimental values for P , plotted against time of recovery.

Moreover, for all substances assayed (including atropine toward acetylcholine) it had a tendency to drop for higher values of R_{50} , as shown in fig. 7 a. The rate of decrease of k' in function of R_{50} is so strikingly similar for so many different inhibitors that it would indicate that the same process develops in the muscle during recovery from inhibition by all antagonists assayed.

It is interesting to refer to a few theoretical implications of formula II. We can easily show that the experimental correlation between R_{50} , determined

directly upon the curves of recovery (the point where the curve crosses the 50 per cent ordinate) and the corresponding average for k' , might be forecast from equation II. In the great majority of the cases, P_0 is too small and can be omitted in face of P . For $P = 50$, the formula is further simplified, giving:

$$k' = 36.1/R_{50} \quad (\text{III})$$

This simple relationship, giving R_{50} directly in seconds, can be graphically represented by a hyperbola, as shown in fig. 7 *a*. Moreover, if, instead of k' , the reciprocal ($\lambda = 1/k'$) is plotted against R_{50} , a straight line is obtained, correlating the increasing values of R_{50} with the increasing values of $1/k'$ (λ). There is a slight tendency of the values of $1/k'$ to deviate from linearity, when they are too small; this is due to the fact that P_0 was omitted in the deduction of equation III and high values of P_0 are connected with high values for constant k' .

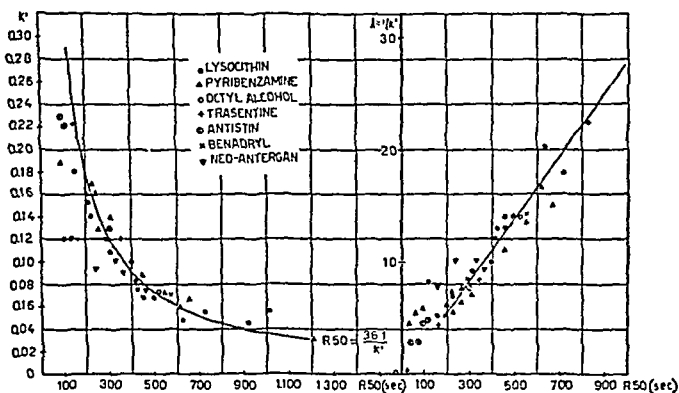


FIG. 7. VALUES OF R_{50} (DETERMINED DIRECTLY UPON EXPERIMENTAL PERCENTAGE-TIME PLOTS), PLOTTED AGAINST AVERAGE k' (LEFT) AND RECIPROCAL OF k' (λ), AT THE RIGHT

Theoretical curves are calculated from equation III

From formula II, it can be deduced that λ , the reciprocal of k' , represents approximately the time for 85 per cent recovery and might *per se* constitute a measure of antagonism. R_{50} , however, is more easily calculated from the curves of recovery, and therefore, by a method independent of k' . The high correlation between R_{50} (found) and k' (average of all values calculated as shown in table 1), clearly seen in figure 7 *a* and *b*, constitutes, therefore, an additional demonstration of the reliability of the method described.

DISCUSSION

A quantitative method for measuring drug antagonism was developed, on the basis of speed of recovery of the guinea pig gut, suspended in a Tyrode solution. This study was further extended to cover a mathematical treatment of the kinetics of the phenomenon and the insufficiency of the accepted theories to explain

this other part of the phenomenon of competition was clearly established. These weak points have been stressed by Clark (12) on the basis that the rate of removal of atropine from the frog's heart is not increased by exposure to high concentrations of acetylcholine. On the other hand, the rate of wash out of the inhibitor is so slow as compared with the rate of wash out of the active drug that one hardly

TABLE 2
Comparative data obtained with different antagonists

TRASENTINE				ANTISTINE			
Dose (γ)	d.f.	$k' \pm SE$	R_{50}	Dose (γ)	d.f.	$k' \pm SE$	R_{50}
5.0	0	0.59	<0	1.0	0	0.75	18"
10.0	0	0.40	<0	2.0	1	0.37 \pm 0.045	42"
100.0	3	0.23 \pm 0.024	162"	3.0	1	0.37 \pm 0.01	60"
300.0	4	0.12 \pm 0.012	354"	5.0	2	0.23 \pm 0.02	96"
				10.0	4	0.16 \pm 0.03	300"

BENADEYL				PYRIBENZAMINE			
Dose (γ)	d.f.	$k' \pm SE$	R_{50}	Dose (γ)	d.f.	$k' \pm SE$	R_{50}
0.5	1	0.37 \pm 0.03	60"	0.02†	2	0.18 \pm 0.011	63"
2.0*	4	0.13 \pm 0.014	300"	0.04†	2	0.17 \pm 0.0024	90"
2.0*	4	0.13 \pm 0.02	288"	0.1*	3	0.16 \pm 0.005	207"
4.0	5	0.098 \pm 0.0043	438"	0.1*	3	0.16 \pm 0.011	225"
5.0*	6	0.070 \pm 0.0035	534"	0.2	3	0.14 \pm 0.010	315"
5.0*	5	0.077 \pm 0.004	570"	0.4	4	0.064 \pm 0.0047	603"
				2.0	7	0.030 \pm 0.001	1200"

NEO-ANTERGAN				LYSOCITHIN			
Dose (γ)	d.f.	$k' \pm SE$	R_{50}	Dose (mg)	d.f.	$k' \pm SE$	R_{50}
0.01†	2	0.13 \pm 0.015	<0	0.33	3	0.14 \pm 0.014	222"
0.02†	3	0.13 \pm 0.017	142"	0.66	5	0.070 \pm 0.0025	428"
0.04†	5	0.10 \pm 0.011	240"	1.00	5	0.070 \pm 0.0014	468"
0.1	5	0.10 \pm 0.006	300"	1.33	6	0.042 \pm 0.0052	624"
0.5	4	0.081 \pm 0.011	468"	1.66	5	0.042 \pm 0.0021	882"
				3.00	7	0.044 \pm 0.0018	912"

* Determinations in the guts of different Guinea pigs.

† The data represent averages of two assays upon the same gut.

d.f. = degrees of freedom in the calculation of SE.

R_{50} represent the values determined upon percentage-time plots.

could imagine that both processes indicate dissociation of reversible compounds with the same receptors. This might, however, indicate that the inhibitor forms a more stable complex with the receptors than the active drug itself. But, even in that case, dissociation of the complex would follow a different course than that experimentally found. In fact, if dissociation of the formed complex is proportional to the initial concentration of the complex formed, regeneration of free

receptors would be maximal at the beginning, slowing down as dissociation proceeds.

We have taken for granted that the percentage of response to a certain, constant dose of histamine added to the external bath, constitutes a quantitative measure of the number of receptors available at the moment of the addition (Gaddum (6)). Therefore, any increase in response is a linear measure of "regeneration" of the receptors, assuming that the recording lever used in these experiments gives approximately linear inscription of the histamine effects (7). The quantitative study of the kinetics of regeneration would induce one to assume that the reappearing receptors would influence or catalyse the process of recovery or, in other words, that regeneration of receptors is an auto-catalytic phenomenon. Any other law of regeneration, as breaking up of an inactive complex of the inhibitor with the receptors, would follow an entirely different curve, as indicated by the increasing values of "constant" k , for a monomolecular reaction.

Analogies with the effects of drugs upon enzymatic systems, led Clark and others to postulate that receptors can be fruitfully represented as patches in the surface of enzymes, the activity of which is essential to the biological response elicited by the drug. Therefore, we might represent receptors for different spasmogenic substances acting upon the same smooth muscle structure as specialized and independent loci upon which the drug is supposed to anchor and act selectively. But owing to the fact that there is no antagonist acting upon one of those receptors to the exclusion of others, but on the contrary all antagonists known up to now, from atropine (the most specific toward acetylcholine) to neo-antergan (the most specific toward histamine) constitute a transition from one extreme to the other, we have to assume that a contiguity or, otherwise, a relationship exists between different receptors for different spasmogenic drugs (Loew (16)).

The fact that exceedingly small doses of inhibitor can block the effect of many equivalents of the active drug has always been a complication in the theoretical interpretation of drug antagonism. If antagonism depends upon a stable combination of the inhibitor with the receptors for the active drug one would expect a great excess of inhibitor to be necessary to block all the receptors available, as it was shown in the case of arginine, histidine and the histamine-aminoacid compounds (2). Even an equimolar ratio would be extremely difficult to interpret on a simple chemical basis. In order to provide an explanation for this difficulty, Pfeiffer (17) has recently developed the theory that the inhibitors might produce an umbrella-like effect protecting or inactivating a larger number of receptors after combining with only one of them.

The facts here described conform with the idea that the inhibitor would, in a certain way, disturb or damage the surface of some enzyme, the activity of which is essential for the response of the muscle. This disturbance or damage would propagate over a more or less extensive area, according to the dose or concentration of the inhibitor applied. According to this view, the role of the specific receptors would be one of directing the effect of the antagonist to preferential loci normally interfered with by the corresponding active drug (histamine or

acetylcholine). It is obvious that if the effect of the antagonist is strong enough the disturbance could propagate to larger areas and other specifically distinct receptors would be partially affected. Recovery from inhibition would represent the auto-catalytic regeneration of the previous status of the enzyme surface.

This view, transferring to the muscle itself its capacity of recovering from inhibition, would explain why regeneration of receptors follows the same law, no matter which inhibitor has been applied. We will show in another paper that certain ions (K^+ and Sr^{++}) accelerate recovery, while Mg^{++} and Ca^{++} slow down the process of recovery. The disturbance postulated above might be, therefore, dependent upon displacement of ionic layers, the integrity of which is essential for muscle contraction (see Szent-Györgyi (18)). That some of the phenomena of ionic interchange at the surface of the myosin molecule can follow an auto-catalytic course was also suggested by Szent-Györgyi.

This explanation, of course, is not the only one possible. To explain the sinusoid curve, one might assume that we are dealing with a variable population of receptors, showing different affinities for the inhibitor, in such a way that at the beginning only the receptors showing less affinity would be liberated, while at the end of the process, those more numerous, showing a medium affinity would appear in a somewhat explosive way. Other possible explanations, as similarity with the process of titrating a buffer solution (in which case pH should be represented by time), are extremely difficult to analyse in concrete terms. None of them, however, would explain all the observed facts as simply as the theory advanced above.

SUMMARY

A method of preparation of lysocithin is described and its inhibitory effect upon the action of histamine, acetylcholine and KCl on the guinea pig gut studied. A simple method for quantitative estimation of lysocithin is described on the basis of the speed of recovery of the gut from inhibition produced by 1 minute contact with lysocithin.

The index R_{50} is defined as the time in seconds for a 50 per cent recovery after washing out of the inhibitor. This index is proposed as a measure of antagonism and it can be correlated to the dose of the inhibitor. A "spectrum" of inhibition can be obtained by testing alternately, upon the same gut, equipotent doses of several spasmogenic agents (histamine, acetylcholine, KCl, etc.).

Application of the method was extended to the antagonism produced by anti-histaminics, as neo-antergan, pyribenzamine, benadryl and antistine and also to antispasmodics of the trasentine type.

The kinetics of the process of recovery from inhibition was studied and a new constant k' was defined as the ratio k/P multiplied by 100, where k represents the "constant" of a first order reaction and P , the percentage contraction at any time, the initial response taken as 100 per cent.

R_{50} was found to be theoretically and experimentally correlated to the average of k' for each concentration of inhibitor, according to the equation of a hyperbola. The reciprocal of k' (λ) is numerically equivalent to the time in minutes for 85

per cent recovery. Since recovery from inhibition followed the same law, no matter which antagonist has been used, the suggestion is made that it depends upon the same intimate process developing in the muscle and that it probably depends upon the auto-catalytic regeneration of a metabolite or of some ionic arrangement in the surface of an operative enzyme, the activity of which is essential for muscle response.

REFERENCES

1. LOEW, E. R., MACMILLAN, R., AND KAISER, M. E.: *This Journal*, 86: 229, 1946.
2. ROCHA E SILVA, M.: *This Journal*, 80: 399, 1944.
3. MORRIS, H. C., AND DRAGSTEDT, C. A.: *Proc. Soc. exp. Biol. and Med.*, 59: 311, 1945.
4. MAYER, R. L., HUTTREY, C. P., AND SCHOLTZ, C. R.: *Science*, 102: 93, 1945.
5. SCHILD, H. O.: *Brit. Journ. Pharm. and Chem.*, 2: 189, 1947.
6. GADDUM, J. H.: In *Modes of drug action*. *Trans. Faraday Soc.*, 39: 323, 1943.
7. SCHILD, H. O.: *Journ. Physiol.*, 103 P, June 3, 1944.
8. KYES, P.: *Z. physiol. Chem.*, 14: 273, 1904.
9. DELEZENNE, C. AND FOURNEAU, E.: *Bull. Soc. chim. France*, 15: 421, 1914.
10. HOUSSAY, B.: *Compt. Rend. Soc. Biol.*, 105: 308, 1930.
11. FELDBERG, W. AND KELLAWAY, C. H.: *Journ. Physiol.* 94: 187, 1938.
12. CLARK, A. J.: In *Heffter's Handbuch exp. Pharmak. Ergaenz.* 4. J. Springer, Berlin, 1937.
13. WELLS, J. A., MORRIS, H. C., BULL, H. B., AND DRAGSTEDT, C. A.: *This Journal*, 85: 122, 1945.
14. LANGMUIR, I.: *Journ. Amer. Chem. Soc.*, 40: 1361, 1918.
15. WINDER, C. V., KAISER, M. E., ANDERSON, M. M., AND GLASSCO, E. M.: *This Journal*, 87: 121, 1946.
16. LOEW, E. R.: *Physiol. Rev.* 27: 542, 1947.
17. PFEIFFER, C. C.: *Science*, 107: 94, 1948.
18. SZENT-GYÖRGYI, A.: *Chemistry of muscular contraction*. Acad. Press Inc., Publishers, New York, 1947.

THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND CENTRAL DEPRESSANT ACTION OF α SUBSTITUTED ETHERS OF GLYCEROL

F. M. BERGER¹

Research Department, The British Drug Houses Ltd., London

Received for publication April 19, 1948

In the past little attention has been paid to the pharmacological properties of α substituted ethers of glycerol. In 1910 Gilbert and Descomps (1) investigated 3-phenoxypropan-1,2-diol and observed that it caused transient paralysis in guinea pigs and rabbits and a sharp but transient fall of body temperature in dogs. The French authors also reported the results of preliminary clinical trials and recommended the substance as an antipyretic and analgesic. It was commercially available under the name of "Antodyne" (2). Berger and Bradley (3, 4) investigated the pharmacological properties of a large number of simple mono ethers of glycerol and found that certain of these compounds possessed a peculiar central depressant action. They suggested that Myanesin, 3-(2'-methylphenoxy) propan-1,2-diol, the most suitable and safest compound of the series, may be useful in the treatment of spastic and hyperkinetic states and for the production of muscular relaxation during anaesthesia. Numerous clinical reports have since shown the usefulness and limitations of the drug in these conditions (5, 6, 7, 8, 9).

The central depressant action of glycerol ethers. The central depressant action of all glycerol ethers possessing this property was, with a few exceptions, qualitatively similar.

After administration of small doses there was little change in the appearance and behaviour of healthy animals. There was a slight decrease in spontaneous activity, but voluntary muscle power and reflexes were not affected. Respiration and blood pressure remained unchanged. In animals showing increased reflexes, similar doses caused a return of reflexes to normal. Experimentally produced tremors and involuntary movements were abolished by similar doses (10). Larger doses caused incoordination and after still larger but tolerated doses there was complete muscular paralysis with a decrease of muscle tone. The posterior half of the animal was more affected than the anterior half and muscles below the costal margin were, as a rule, more flaccid, and remained relaxed for a longer period of time than those above the costal margin. Spontaneous respiration was maintained even during profound paralysis indicating that the diaphragm was not affected. The corneal reflex and consciousness were not lost. Paralysis, as a rule, was neither preceded nor followed by excitation. Decerebrate rigidity could be abolished and tetanic spasms relieved. Administration of these substances prior to ether or barbiturate anaesthesia caused suppression

¹ Present address University of Rochester, School of Medicine and Dentistry, Rochester 7, New York.

of the stage of excitation and a smoother narcosis. The effects of strychnine and metrazol could be antagonized but nikethamide and picrotoxin did not possess antagonistic properties (11). Numerous compounds of this series prevented the occurrence of electrically induced convulsions. Lethal doses caused death by respiratory paralysis.

I. THE PARALYZING ACTION OF GLYCEROL ETHERS

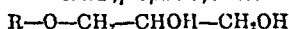
METHOD. Adult male white mice weighing 18 to 23 grams were used. On the average about 60 mice in groups of 10 to 15 were used at different dose levels for each drug. The loss of the righting reflex for at least one minute was taken as criterion of paralysis. Deaths were counted 24 hours after administration of the drug. The median paralyzing and lethal doses, found graphically by the method of Miller and Tainter (12), were used as indices of the activity and toxicity of the compounds. Because of the low water solubility of many of the substances, suspensions in gum acacia 5 per cent w/v were prepared and administered subcutaneously. A few substances which were ineffective when administered in this manner were given intraperitoneally dissolved in 10 per cent v/v of aqueous propylene glycol. The total amount of propylene glycol administered in this way never exceeded 5 cc. per kg. body weight. This amount did not cause any symptoms when injected by itself. The mean lethal dose of the sample of propylene glycol used was 14.5 ± 1.5 cc. per kg. body weight for mice after intraperitoneal administration. The results were expressed in millimols per kg. body weight.

1) *3-Alkoxypropan-1,2-diols* (table 1). When the alkyl group was methyl, ethyl, and propyl the compounds did not show any paralyzing or toxic effects in the doses tried. The n-butyl derivative possessed paralyzing action. The maximum activity was shown by the n-amyl compound. A further increase in the length of the hydrocarbon chain reduced activity. The iso-butyl and iso-amyl ethers were less active than the corresponding normal isomers. The allyl ether did not show activity but was more toxic than the corresponding saturated ether. The 2-methyl allyl compound was also inactive, but the introduction of a bromine atom into the allyl radical caused the re-appearance of paralyzing activity.

2) *3-Aryloxypropan-1,2-diols* (table 2). The 3-phenoxypropan-1,2-diol had greater paralyzing activity than most members of the aliphatic series. The introduction of an amino group into the nucleus abolished the paralyzing activity and the paralyzing action could not be restored by acetylation of the amino group. The 3-(4'-propionylamidophenoxy) propan-1,2-diol, however, was active. The 2- and 4-formylphenyl ethers were devoid of paralyzing action, but the 3-nitrophenyl ether possessed some activity. The benzyl ether had stronger paralyzing action and was relatively less toxic than the phenyl ether. Alkyl and alkoxy substitution in ortho position in the benzyl nucleus increased both activity and toxicity, but the increase in toxicity was relatively larger than the gain of paralyzing action. The saturated homologue of the phenyl ether, the cyclohexyl ether did not possess any paralyzing action but had convulsant properties. The α and β -naphthyl ethers were devoid of paralyzing action in non-lethal doses. The 2-quinolyl and the 8-(1,2,3,4-tetrahydro)quinolyl ethers had marked paralyzing action and a distinct margin of safety. The 8-quinolyl ether after sub-

TABLE 1

3-Alkoxypropan-1,2-diol



Mean paralyzing and lethal doses of 3-alkoxypropan-1,2-diols in millimols per kg. body weight after subcutaneous administration to white mice

R-O-CH ₂ -CHOH-CH ₂ OH R-SUBSTITUENT	MOLECULAR WEIGHT	PD ₅₀ ± SE	LD ₅₀ ± SE	LD ₅₀ PD ₅₀
		mM/kg.	mM/kg.	
Methyl	106	>24.0	>24.0	—
Ethyl	120	>21.0	>21.0	—
n-Propyl	134	>15.4	>15.4	—
n-Butyl ..	148	10.0 ± 0.36	18.9 ± 1.0	1.9
Isobutyl	148	10.8 ± 1.1	>17.0	>1.6
n-Amyl ..	162	5.4 ± 0.3	12.3 ± 0.62	2.3
Isomyl	162	7.6 ± 0.49	13.0 ± 0.8	1.7
n-Hexyl	176	6.0 ± 0.4	12.7 ± 0.46	2.1
Cetyl ..	316	>9.5	>9.5	—
Octadecyl ..	344	>8.8	>8.8	—
Allyl ..	132	—	8.6 ± 0.68	—
2-Methylallyl	146	—	11.1 ± 1.0	—
1-Bromallyl ..	211.9	7.6 ± 0.71	9.8 ± 0.85	1.3
1-Brom-2-methylallyl	224.9	3.1 ± 0.35	4.3 ± 0.37	1.4

TABLE 2

3-Aryloxypropan-1,2-diols

Mean paralyzing and lethal doses of 3-aryloxypropan-1,2-diols after subcutaneous administration to white mice

R-O-CH ₂ -CHOH-CH ₂ OH R-SUBSTITUENT	MOLECULAR WEIGHT	PD ₅₀ ± SE	LD ₅₀ ± SE	LD ₅₀ PD ₅₀
		mM/kg	mM/kg.	
Phenyl	168	5.5 ± 0.41	10.0 ± 0.56	1.8
2-Aminophenyl	183	—	7.7 ± 0.55	—
4-Aminophenyl ..	183	—	>16.3	—
2-Acedamidophenyl ..	213	—	7.7 ± 0.3	—
4-Acedamidophenyl ..	213	—	12.0 ± 0.79	—
4-Propionylamidophenyl ..	239	5.6 ± 0.45	8.0 ± 0.59	1.4
2-Methyl-4-aminophenyl ..	197	—	16.3 ± 1.12	—
4-(2'Amino-2'-carboxy)ethyl- phenyl (tyrosyl)	255	—	8.2 ± 0.62	—
2-Formylphenyl ..	196	—	5.8 ± 0.37	—
4-Formylphenyl ..	196	—	12.8 ± 0.94	—
3-Nitrophenyl ..	213	2.9 ± 0.1	4.0 ± 0.29	1.4
Benzyl ..	182	3.3 ± 0.12	10.0 ± 0.6	3.0
4-Methylbenzyl ..	196	1.6 ± 0.19	3.5 ± 0.36	2.2
4-Ethylbenzyl ..	210	1.6 ± 0.14	4.5 ± 0.27	2.8
4-Methoxybenzyl ..	212	5.4 ± 0.45	11.3 ± 0.75	2.1
2-Ethoxybenzyl ..	226	1.5 ± 0.11	4.2 ± 0.28	2.8
4-Ethoxybenzyl ..	226	—	7.7 ± 0.53	—
Cyclohexyl ..	174	—	2.0 ± 0.17	—
α-Naphthyl ..	218	—	3.8 ± 0.25	—
β-Naphthyl ..	218	—	8.7 ± 0.64	—
2-Quinolyl ..	219	1.2 ± 0.05	3.4 ± 0.21	2.8
8-Quinolyl ..	219	1.1* ± 0.05	2.5* ± 0.14	2.3
8-(1,2,3,4-tetrahydro)quinolyl	233	1.8 ± 0.19	1.8 ± 0.18	2.6

* Intraperitoneal administration

cutaneous administration caused drowsiness without loss of the righting reflex. After intraperitoneal administration complete paralysis was observed. The quaternary salt, the 8-(2',3'-dihydroxypropoxy)-1,1-dimethyl-1,2,3,4-tetrahydroquinolinium iodide possessed typical curare-like action but did not have any paralyzing action in tolerated doses. The mean lethal dose on intraperitoneal administration to mice was 2.5 ± 0.3 mM. per kg.

3) *Nuclear substitution of 8-phenoxypropan-1,2-diol* (table 3).

a) *Alkyl substitution*. Introduction of a single methyl or ethyl group into the nucleus markedly increased the paralyzing activity. The toxicity of these compounds was also increased but relatively less so. The position of the alkyl group in the nucleus was of great importance. Compounds with methyl or ethyl groups in ortho position were markedly more active than isomers with the same group in the meta or para position. They were also more toxic but had a wider margin of safety than the corresponding para isomers. Substitution of an n-propyl group in the ortho or the para position did not influence activity but somewhat increased toxicity, the para compound being more toxic than the ortho isomer. The 4-n-butylphenyl ether was less active than the unsubstituted phenyl ether and had paralyzing action in nearly lethal doses only. The 2-allylphenyl ether was more toxic than any of the saturated compounds and possessed but little paralyzing activity in non-lethal doses.

Compounds with two alkyl groups in the nucleus had greater activity with the substituent groups in the 2,6 position than with groups in the 2,4 or the 2,5 position. When one of the groups was unsaturated the activity, as compared with that of the corresponding saturated compound, was increased.

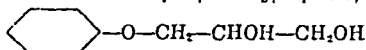
The effects of nuclear substitution with alkoxy groups were similar to those observed with alkyl substitution. The 2-methoxy, 2-ethoxy, 2-propoxy and 2-butoxy compounds produced paralysis in similar or smaller doses as the 2-methyl and 2-ethyl compounds and also possessed similar toxicity. These alkoxy compounds differed, however, from the corresponding alkyl compounds in causing some excitation prior to paralysis. This was thought to be due to a more marked depressant effect on the higher centra. The 4-methoxy ether was much less active than the ortho isomer. It was also less toxic but the decrease of toxicity was not proportional to the loss of paralyzing activity.

Compounds with a hydroxy radical attached to the ring directly or through an additional carbon atom did not possess paralyzing activity.

3b) *Halogen substitution*. The effect of chlorine and bromine substitution depended on its relative position in the nucleus in the same way as when alkyl or alkoxy groups were the substituent. The 2-chloro and 2-bromo compounds again possessed greater activity and a wider margin of safety than the 4-chloro and 4-bromo compounds. Bromination as compared with chlorination causes a decrease in activity without a corresponding diminution of toxicity. The iodo compound appeared to be still less active but could not be satisfactorily tested because of its very low solubility and the rapid separation of suspensions in gum acacia.

The introduction of two or more chlorine atoms into the nucleus decreased activity. The 2,4,6-trichloro compound did not possess any paralyzing activity.

TABLE 3
Nuclear substitution of 3-phenoxypropan-1,2-diol



Mean paralyzing and lethal doses after subcutaneous administration to white mice

CLASS	SUBSTITUENT	MOLECULAR WEIGHT	PD ₅₀ ± SE	LD ₅₀ ± SE	LD ₅₀ /PD ₅₀
			<i>mM/kg.</i>	<i>mM/kg.</i>	
Alkyl	none	168	5.5 ± 0.58	10.0 ± 0.56	1.8
	2-methyl	182	1.8 ± 0.11	5.5 ± 0.31	3.0
	3-methyl	182	3.1 ± 0.28	8.1 ± 0.49	2.6
	4-methyl	182	2.9 ± 0.21	7.0 ± 0.33	2.4
	2-ethyl	196	1.8 ± 0.19	6.4 ± 0.4	3.5
	4-ethyl	196	4.2 ± 0.2	7.4 ± 0.34	1.8
	2-n-propyl	210	5.8 ± 0.51	9.2 ± 0.63	1.6
	4-n-propyl	210	5.3 ± 0.38	6.9 ± 0.31	1.3
	4-n-butyl	224	8.0 ± 0.44	8.7 ± 0.46	1.1
	2-allyl	208	3.1 ± 0.3	3.6 ± 0.26	1.2
2-Alkyls	2,4-dimethyl	196	>5.1	>5.1	—
	2-methyl-6-n-propyl	224	5.0 ± 0.46	6.5 ± 0.56	1.3
	2-methyl-6-allyl	222	2.8 ± 0.27	4.5 ± 0.4	1.6
	2-n-propyl-4-methyl	224	8.3 ± 0.5	13.6 ± 0.63	1.6
	2-allyl-4-methyl	222	6.7 ± 0.49	11.7 ± 0.85	1.8
	2-i-propyl-5-methyl	224	8.3 ± 0.5	10.5 ± 0.92	1.3
	2-methyl-5-i-propyl	224	—	4.0 ± 0.39	—
	2-methoxy	198	1.4 ± 0.09	5.0 ± 0.33	3.5
Alkoxy	3-methoxy	198	2.5 ± 0.21*	6.8 ± 0.36*	2.7
	4-methoxy	198	4.7 ± 0.25	8.1 ± 0.37	1.7
	2-ethoxy	212	1.4 ± 0.12	5.2 ± 0.25	3.8
	4-ethoxy	212	2.4 ± 0.16	3.5 ± 0.28	1.5
	2-propoxy	226	1.1 ± 0.04	4.0 ± 0.26	3.6
	2-butoxy	240	1.5 ± 0.08	3.0 ± 0.15	2.0
	2-heptoxy	284	—	>7.4	—
	2-allyloxy	224	1.1 ± 0.05	2.6 ± 0.28	2.4
Hydroxy	2-hydroxy	184	—	6.5 ± 0.4	—
	4-hydroxy	184	—	9.8 ± 0.82	—
Hydroxy alkyl	2-hydroxymethyl	198	—	7.1 ± 1.1	—
Halogen	2-chloro	202.5	1.7 ± 0.19	4.5 ± 0.25	2.6
	4-chloro	202.5	2.1 ± 0.23	4.5 ± 0.41	2.2
	2-bromo	247	2.1 ± 0.19	4.7 ± 0.32	2.2
	4-bromo	247	3.4 ± 0.18	4.7 ± 0.23	1.4
	2,4-dichloro	237	2.3 ± 0.22	3.5 ± 0.18	1.5
	2,4,6-trichloro	271.5	—	6.0 ± 0.29	—
	3-methyl-4-chloro	216.5	4.0 ± 0.4	8.0 ± 0.34	2.0
Alkyl and Halogen	3,5-dimethyl-4-chloro	230.5	6.1 ± 0.37	10.1 ± 0.61	1.6
	2-chloro-6-n-propyl	244.5	8.0 ± 0.7	10.0 ± 0.81	1.2
	2-n-propyl-4-chloro	244.5	8.4 ± 0.81	11.2 ± 0.9	1.3
	2-allyl-4-chloro	242.5	5.8 ± 0.45	8.2 ± 0.7	1.4
	2-chloro-6-allyl	242.5	5.6 ± 0.5	7.0 ± 0.75	1.3
	2-n-propyl-4-bromo	289	8.6 ± 0.64	>8.6	—
	2-bromo-6-n-propyl	289	4.9 ± 0.38	6.6 ± 0.44	1.3
	2-bromo-6-allyl	287	5.9 ± 0.46	8.0 ± 0.66	1.3
	2-allyl-4-bromo	287	5.2 ± 0.49	7.0 ± 0.52	1.3

* Intraperitoneal administration

Compounds having both a halogen and alkyl group in the ring were relatively non-toxic and possessed but little paralyzing activity in non-lethal doses. When the alkyl group was unsaturated, paralyzing activity was increased in chlorinated compounds but not in brominated compounds. Substances with a methyl group in ortho position were not investigated and it is possible that such compounds would have had a stronger paralyzing action.

4) *Derivatives of 3-acylphenoxypropan-1,2-diol* (table 4). The 4-carboxyphenyl ether did not possess any paralyzing activity. The 2-carbomethoxyphenyl ether showed some paralyzing action in large doses but most esters and amides of the carboxy phenyl ethers were devoid of paralyzing activity. The ketonic derivatives possessed paralyzing activity when the group was in ortho position but were devoid of activity when similar groups were in para position.

TABLE 4

Acyl derivatives of 3-phenoxypropan-1,2-diol

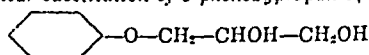
Mean paralyzing and lethal doses after subcutaneous administration to white mice

CLASS	SUBSTITUENT ON PHENYL GROUP	MOLECULAR WEIGHT	PD ₁₀ ± SE	LD ₁₀ ± SE	LD ₁₀ / PD ₁₀
			mM/kg.	mM/kg.	
Acid	4-carboxy	212	—	11.8 ± 0.9	—
Esters	2-carbomethoxy	226	7.8 ± 0.67	14.2 ± 0.89	1.8
	4-carboethoxy	240	—	7.3 ± 0.6	—
	4-carbo-n-propoxy	254	—	11.4 ± 0.81	—
	4-carbo-n-butoxy	268	—	>9.3	—
Amides	2-carbamyl	211	—	14.5 ± 0.53	—
Ketones	2-acetyl	210	6.2 ± 0.57	12.2 ± 0.86	2.0
	4-acetyl	210	—	8.0 ± 0.47	—
	2-propionyl	224	3.4 ± 0.25	5.8 ± 0.47	1.7
	4-propionyl	224	—	3.4 ± 0.36	—
	2-n-butyryl	238	3.4 ± 0.41	8.0 ± 0.45	2.3
	4-n-butyryl	238	—	3.2 ± 0.29	—
Oximes of	4-acetyl	225	—	6.8 ± 0.43	—
	4-n-butyryl	253	—	8.2 ± 0.59	—

The compounds with ketonic groups in the para position were also considerably more toxic than the corresponding ortho compounds, the para C₃ and C₄ ketone being more than twice as toxic as the para C₂ ketone. The oximes of the C₂, C₃ and C₄ ketones did not have paralyzing action.

5) *Branched chain compounds* (tables 5). Compounds with a methyl group on the C₂ atom of the glyceryl chain of the general formula aryl-O-CH₂-C(CH₃)-OH-CH₂OH possessed paralyzing properties equal or superior to the corresponding straight chain ethers without the methyl group. They showed similar relationships between nuclear substitution and paralyzing activity as the straight chain compounds. The phenyl ether of the branch chain series was very much more active and relatively less toxic than the corresponding straight chain ether. Nuclear substitution of the branched chain ethers apparently reduced activity when suspensions of the compounds in gum acacia were injected subcutaneously.

TABLE 3
Nuclear substitution of 3-phenoxypropan-1,2-diol



Mean paralyzing and lethal doses after subcutaneous administration to white mice

CLASS	SUBSTITUENT	MOLECULAR WEIGHT	PD ₅₀ ± SE	LD ₅₀ ± SE	LD ₅₀ PD ₅₀
			mM/kg.	mM/kg	
Alkyl	none	168	5.5 ± 0.58	10.0 ± 0.56	1.8
	2-methyl	182	1.8 ± 0.11	5.5 ± 0.31	3.0
	3-methyl	182	3.1 ± 0.28	8.1 ± 0.49	2.6
	4-methyl	182	2.9 ± 0.21	7.0 ± 0.33	2.1
	2-ethyl	196	1.8 ± 0.19	6.4 ± 0.4	3.5
	4-ethyl	196	4.2 ± 0.2	7.4 ± 0.34	1.8
	2-n-propyl	210	5.8 ± 0.51	9.2 ± 0.63	1.6
	4-n-propyl	210	5.3 ± 0.38	6.9 ± 0.31	1.3
	4-n-butyl	224	8.0 ± 0.44	8.7 ± 0.46	1.1
	2-allyl	208	3.1 ± 0.3	3.6 ± 0.26	1.2
2-Alkyls	2,4-dimethyl	196	>5.1	>5.1	—
	2-methyl-6-n-propyl	224	5.0 ± 0.46	6.5 ± 0.56	1.3
	2-methyl-6-allyl	222	2.8 ± 0.27	4.5 ± 0.4	1.6
	2-n-propyl-4-methyl	224	8.3 ± 0.5	13.6 ± 0.63	1.6
	2-allyl-4-methyl	222	6.7 ± 0.49	11.7 ± 0.85	1.8
	2-i-propyl-5-methyl	224	8.3 ± 0.5	10.5 ± 0.92	1.3
	2-methyl-5-i-propyl	224	—	4.0 ± 0.39	—
	2-methoxy	198	1.4 ± 0.09	5.0 ± 0.33	3.5
	3-methoxy	198	2.5 ± 0.21*	6.8 ± 0.36*	2.7
	4-methoxy	198	4.7 ± 0.25	8.1 ± 0.37	1.7
Alkoxy	2-ethoxy	212	1.4 ± 0.12	5.2 ± 0.25	3.8
	4-ethoxy	212	2.4 ± 0.16	3.5 ± 0.28	1.5
	2-propoxy	226	1.1 ± 0.04	4.0 ± 0.26	3.6
	2-butoxy	240	1.5 ± 0.08	3.0 ± 0.15	2.0
	2-heptoxy	284	—	>7.4	—
	2-alkoxy	224	1.1 ± 0.05	2.6 ± 0.28	2.4
	2-hydroxy	184	—	6.5 ± 0.4	—
	4-hydroxy	184	—	9.8 ± 0.82	—
	2-hydroxymethyl	198	—	7.1 ± 1.1	—
	2-chloro	202.5	1.7 ± 0.19	4.5 ± 0.25	2.6
Halogen	4-chloro	202.5	2.1 ± 0.23	4.5 ± 0.41	2.2
	2-bromo	247	2.1 ± 0.19	4.7 ± 0.32	2.2
	4-bromo	247	3.4 ± 0.18	4.7 ± 0.23	1.4
	2,4-dichloro	237	2.3 ± 0.22	3.5 ± 0.18	1.5
	2,4,6-trichloro	271.5	—	6.0 ± 0.29	—
	3-methyl-4-chloro	216.5	4.0 ± 0.4	8.0 ± 0.34	2.0
	3,5-dimethyl-4-chloro	230.5	6.1 ± 0.37	10.1 ± 0.61	1.6
	2-chloro-6-n-propyl	244.5	8.0 ± 0.7	10.0 ± 0.81	1.2
	2-n-propyl-4-chloro	244.5	8.4 ± 0.81	11.2 ± 0.9	1.3
	2-allyl-4-chloro	242.5	5.8 ± 0.45	8.2 ± 0.7	1.4
Alkyl and Halogen	2-chloro-6 allyl	242.5	5.6 ± 0.5	7.0 ± 0.75	1.3
	2-n-propyl-4-bromo	289	8.6 ± 0.64	>8.6	—
	2-bromo-6-n-propyl	289	4.9 ± 0.38	6.6 ± 0.44	1.3
	2-bromo-6-allyl	287	5.9 ± 0.46	8.0 ± 0.66	1.3
	2-allyl-4-bromo	287	5.2 ± 0.49	7.0 ± 0.52	1.3

* Intraperitoneal administration

Compounds having both a halogen and alkyl group in the ring were relatively non-toxic and possessed but little paralyzing activity in non-lethal doses. When the alkyl group was unsaturated, paralyzing activity was increased in chlorinated compounds but not in brominated compounds. Substances with a methyl group in ortho position were not investigated and it is possible that such compounds would have had a stronger paralyzing action.

4) *Derivatives of 3-acylphenoxypropan-1,2-diol* (table 4). The 4-carboxyphenyl ether did not possess any paralyzing activity. The 2-carbomethoxyphenyl ether showed some paralyzing action in large doses but most esters and amides of the carboxy phenyl ethers were devoid of paralyzing activity. The ketonic derivatives possessed paralyzing activity when the group was in ortho position but were devoid of activity when similar groups were in para position.

TABLE 4

Acyl derivatives of 3-phenoxypropan-1,2-diol

Mean paralyzing and lethal doses after subcutaneous administration to white mice

CLASS	SUBSTITUENT ON PHENYL GROUP	MOLECULAR WEIGHT	PD ₅₀ ± SE	LD ₅₀ ± SE	LD ₅₀ /PD ₅₀
			<i>mM/kg</i>	<i>mM/kg</i>	
Acid	4-carboxy	212	—	11.8 ± 0.9	—
Esters	2-carbomethoxy	226	7.8 ± 0.67	14.2 ± 0.89	1.8
	4-carboethoxy	240	—	7.3 ± 0.6	—
	4-carbo-n-propoxy	254	—	11.4 ± 0.81	—
	4-carbo-n-butoxy	268	—	>9.3	—
Amides	2-carbamyl	211	—	14.5 ± 0.53	—
Ketones	2-acetyl	210	6.2 ± 0.57	12.2 ± 0.86	2.0
	4-acetyl	210	—	8.0 ± 0.47	—
	2-propionyl	224	3.4 ± 0.25	5.8 ± 0.47	1.7
	4-propionyl	224	—	3.4 ± 0.36	—
	2-n-butyryl	238	3.4 ± 0.41	8.0 ± 0.45	2.3
	4-n-butyryl	238	—	3.2 ± 0.29	—
Oximes of	4-acetyl	225	—	6.8 ± 0.43	—
	4-n-butyryl	253	—	8.2 ± 0.59	—

The compounds with ketonic groups in the para position were also considerably more toxic than the corresponding ortho compounds, the para C₂ and C₄ ketone being more than twice as toxic as the para C₂ ketone. The oximes of the C₂, C₃ and C₄ ketones did not have paralyzing action.

5) *Branched chain compounds* (tables 5). Compounds with a methyl group on the C₂ atom of the glyceryl chain of the general formula aryl-O-CH₂-C(CH₃)-OH-CH₂OH possessed paralyzing properties equal or superior to the corresponding straight chain ethers without the methyl group. They showed similar relationships between nuclear substitution and paralyzing activity as the straight chain compounds. The phenyl ether of the branch chain series was very much more active and relatively less toxic than the corresponding straight chain ether. Nuclear substitution of the branched chain ethers apparently reduced activity when suspensions of the compounds in gum acacia were injected subcutaneously.

After intraperitoneal administration of solutions in aqueous propylene glycol these methyl phenyl ethers were more active than the phenyl ether and substitution in the ortho position gave compounds of greater paralyzing activity than substitution in the meta or para position. The discrepancy between the results after administration of compounds in suspension and solution may be due to the low solubility of the branched chain compounds. The 3-(4'-methylphenoxy)-2-methylpropan-1,2-diol one of the least soluble compounds did not cause complete paralysis after as much as 2000 mg. per kg. (10.2 mM/kg.) when administered subcutaneously in a suspension in gum acacia, but paralyzed mice

TABLE 5

Branched chain compounds

Mean paralyzing and lethal doses of 3-aryloxypropan-1,2-diols substituted with a methyl group on the C₁ or C₂ carbon, after subcutaneous administration to white mice

R ₁ -O-CH ₂ -CH(OH)-CH ₂ OH			MOLECULAR WEIGHT	PD ₅₀ ± SE	LD ₅₀ ± SE	LD ₅₀ /PD ₅₀
R ₁	R ₂	R ₃				
				mM/kg.	mM/kg.	
Phenyl	methyl	H	182	1.9 ± 0.14	7.1 ± 0.69	3.4
2-methylphenyl	methyl	H	196	1.5* ± 0.15	5.4* ± 0.5	3.6
				2.1 ± 0.33	4.6 ± 0.7	2.2
3-methylphenyl	methyl	H	196	0.92* ± 0.05	2.9* ± 0.18	3.2
				2.8 ± 0.43	5.6 ± 0.69	2.0
4-methylphenyl	methyl	H	196	1.1* ± 0.05	3.2* ± 0.12	2.9
3,4-dimethylphenyl	methyl	H	210	1.3* ± 0.05	4.0* ± 0.19	3.2
3,5-dimethylphenyl	methyl	H	210	7.8 ± 0.83	10.7 ± 0.64	1.4
2-methoxyphenyl	methyl	H	212	3.1 ± 0.29	8.1 ± 0.34	2.6
3-methoxyphenyl	methyl	H	212	1.6 ± 0.25	5.8 ± 0.29	3.5
4-methoxyphenyl	methyl	H	212	1.5* ± 0.04	5.4* ± 0.33	3.7
2-chlorophenyl	methyl	H	216.5	5.3 ± 0.45	9.2 ± 0.61	1.7
4-chlorophenyl	methyl	H	216.5	3.5 ± 0.03	5.4 ± 0.13	1.5
3-methyl-4-chlorophenyl	methyl	H	230.5	4.8 ± 0.34	5.7 ± 0.42	1.2
4-acetamidophenyl	methyl	H	227	—	5.6 ± 0.54	—
phenyl	H	methyl	182	9.3 ± 0.99	11.3 ± 0.95	1.2
4-methylphenyl	H	methyl	196	>10.7	>10.7	—

* Intraperitoneal administration.

after 250 mg. per kg. (1.3 mM/kg.) when administered intraperitoneally in aqueous propylene glycol solution.

Nuclear substitution with two methyl groups decreased activity. The presence of a methoxy group in the ortho position gave a very active compound markedly superior to the para isomer, and the same relation applied after nuclear substitution with chlorine in the ortho and para position.

Compounds with a methyl group on the C₁ atom with the general formula aryl-O-CH₂-CHOH-CH(CH₃)OH possessed but little paralyzing activity. They were difficult to test because of their insolubility in water and aqueous propylene glycol.

6) *Substitution in the hydroxy radicals of the glyceryl side chain* (table 6). The diacetates of allyl, 2-methylphenyl and benzyl ethers and the glucosides of 2-methylphenyl and 4-chlorophenyl ethers possessed but little paralyzing activity. Two compounds with substituents in the C₁ and C₃ hydroxyl groups were also examined. They were 3-(2'-methylphenoxy)-1-methoxy-propan-2-ol and 3-(2'-methylphenoxy)-1-benzyloxypropan-2-ol. Both of them were devoid of paralyzing action in non-lethal doses. After lethal doses they caused ex-

TABLE 6

Substitution in hydroxyl radicals

Mean paralyzing and lethal dose after subcutaneous administration to white mice

R ₁ -O-CH ₂ -CHOR ₂ -CH ₂ OR ₃			MOLECULAR WEIGHT	PD ₅₀ ± SE	LD ₅₀ ± SE	LD ₅₀ PD ₅₀
R ₁	R ₂	R ₃				
				mM/kg.	mM/kg.	
Allyl	acetyl	acetyl	216	—	8.1 ± 0.77	—
2-methylphenyl	acetyl	acetyl	266	—	>7.5	—
benzyl	acetyl	acetyl	266	8.7 ± 0.6	9.8 ± 0.81	1.1
2-methylphenyl	H	glucosido	344	—	>7.3	—
4-chlorophenyl	H	glucosido	364.5	—	4.1 ± 0.45	—
2-methylphenyl	H	methyl	196	—	2.5 ± 0.2	—
2-methylphenyl	H	benzyl	272	—	>10.5	—
2-methylphenyl	H	dipotassium phosphate	337	3.0 ± 0.22	4.1 ± 0.35	1.4

TABLE 7

The influence of various linkages between the nucleus and the glyceryl side chain

Mean paralyzing and lethal doses after intraperitoneal administration to white mice

R ₁ -R ₂ -CH ₂ -CHOH-CH ₂ OH		MOLECULAR WEIGHT	PD ₅₀ ± SE	LD ₅₀ ± SE	LD ₅₀ PD ₅₀
R ₁	R ₂				
			mM/kg.	mM/kg.	
4-methylphenyl	O	182	1.9 ± 0.08	5.6 ± 0.26	3.0
4-methylphenyl	S	198	1.2 ± 0.08	3.4 ± 0.22	2.8
4-methylphenyl	SO ₂	230	2.2 ± 0.09	3.7 ± 0.21	1.7
4-methylphenyl	NH	181	—	1.1 ± 0.03	—

citation which was followed by paralysis. Dipotassium 3-(2'-methylphenoxy)-2-hydroxypropyl phosphate had some paralyzing action.

7) *The linkage between nucleus and side chain* (table 7). The significance of the ether linkage between the aryl radical and the glyceryl side chain was also investigated. Compounds with a sulphur or sulphone linkage, such as 3-(4'-methylphenylthio)-propan-1,2-diol and 3-(4'-methylphenylsulphonyl)-propan-1,2-diol possessed paralyzing activity. The thio ether was more active and the sulphone less active, but both compounds were more toxic than the corresponding oxygen ether. The 3-(4'-methylphenylamino)-1-propan-1,2-diol was devoid of

TABLE 8

The anticonvulsant action of S-aryloxypropan-1,2-diols and S-arylthioprop-1,2-diols after oral administration to rats convulsed by an interrupted electric current

NO.	R-CH ₂ -CHOH-CH ₂ -OH SUBSTITUENT R	DOSE ORALLY	RESPONSE*	MEAN ELEVATION OF THRESHOLD	PD ₅₀ ± SE IN MICE INTRA- PERITONEALLY	LD ₅₀ ± SE IN MICE INTRA- PERITONEALLY
		mg./kg.		volts	mg./kg.	mg./kg.
1	Phenoxy	400	3/4	75 ± 15	420 ± 14	1240 ± 93
2	2'-methylphenoxy	400	4/4	36 ± 18	180 ± 7	600 ± 16
		200	0/4	0		
3	3'-methylphenoxy	400	4/4	73 ± 2	255 ± 9	750 ± 30
		200	2/4	61 ± 4		
4	4'-methylphenoxy	400	4/4	45 ± 18	340 ± 15	1020 ± 48
		200	2/4	12 ± 2		
5	2'-ethylphenoxy	400	4/1	85 ± 3	255 ± 9	560 ± 15
		200	7/8	35 ± 2		
6	4'-ethylphenoxy	400	4/4	54 ± 18	820† ± 38	1450† ± 67
		200	3/4	17 ± 4		
7	2'-methoxyphenoxy	400	4/4	58 ± 15	365 ± 11	1160 ± 51
		200	2/4	10 ± 2		
8	3'-methoxyphenoxy	400	3/4	50 ± 5	495 ± 41	1350 ± 72
		200	2/4	45 ± 10		
9	4'-methoxyphenoxy	400	3/4	76 ± 19	745 ± 60	1650 ± 99
		200	2/4	55 ± 20		
10	2'-ethoxyphenoxy	400	4/1	90 ± 3	250 ± 7	800 ± 35
		200	4/4	37 ± 14		
11	4'-ethoxyphenoxy	400	4/4	61 ± 25	412 ± 15	740 ± 35
		200	2/4	20 ± 5		
12	2'-propoxyphenoxy	400	4/4	90 ± 5	260 ± 12	650 ± 54
		200	3/4	25 ± 3		
13	2'-butoxyphenoxy	400	4/4	77 ± 10	216 ± 22	710 ± 24
		200	3/4	13 ± 4		
14	2'-heptoxyphenoxy	400	4/4	57 ± 9	>2100†	>2100†
		200	2/4	17 ± 10		
15	2'-alloxyphenoxy	400	4/4	60 ± 14	150 ± 11	650 ± 55
		200	2/4	17 ± 7		
16	Phenylthio	400	4/4	20 ± 10	280 ± 30	940 ± 95
17	2'-methylphenylthio	400	4/4	95 ± 8	210 ± 11	600 ± 30
		200	3/4	63 ± 15		
18	4'-methylphenylthio	400	2/4	60 ± 30	240 ± 15	680 ± 43
19	2'-methoxyphenylthio	400	2/4	60 ± 5	340 ± 26	>940
20	4'-methoxyphenylthio	400	4/4	47 ± 11	510 ± 42	>940
		200	0/4			
21	Tridione	600	4/4	81 ± 8	1750 ± 100	2400 ± 180
		400	1/4	65		
		200	0/4			

* Response $\frac{\text{Number of animals showing an increase in threshold}}{\text{number of animals tested}}$

† Subcutaneous administration in gum acacia.

paralyzing activity. It was more toxic than any of the oxygen ethers and possessed convulsant properties.

II. THE ANTICONVULSANT PROPERTY OF GLYCEROL ETHERS

1) *The antagonism to metrazol.* The effect of compounds No. 1,2,4,5,7 and 9 of table 8 on convulsions produced by metrazol was examined in mice. The compounds were administered intraperitoneally and after a period of 15 minutes metrazol 100 mg. per kg. was injected intravenously. The injection of metrazol was spread over one minute. All control animals treated with metrazol only convulsed and about one half of them died. The various compounds prevented the occurrence of convulsions and death when administered in paralyzing doses, but had little or no effect in smaller doses. Tridione, on the other hand suppressed convulsions in doses of 600 mg. per kg. which was about one third of the dose causing loss of the righting reflex.

2) *The effect of drugs on electrically induced convulsions.* Convulsions were induced in rats by a square wave current of a frequency of 100 cycles per second, administered for 2 seconds. The electrodes were placed at the occiput and in the mouth of the animals. At intervals of 10 or more minutes current of increasing strength was applied until the convulsive threshold was found. The drugs were then administered orally in a suspension of gum acacia and the threshold re-examined at intervals for 3 hours. The highest elevation of threshold obtained with various doses within this period of time is given in table 8. The table also gives the mean paralyzing and lethal doses of the compounds after intraperitoneal administration of aqueous solutions to mice.

All the compounds examined elevated the threshold to electric convulsions to a similar extent and there was no correlation between anticonvulsive and paralytic activities of the substances. For instance, compounds No. 2 and 10, which possessed considerable paralytic activity and caused incoordination and marked depression of spontaneous activity in rats after doses of 400 mg. per kg., did not cause a significantly greater reduction of the irritability of the cortex to electric current than compounds No. 9 and 14 which possessed but little paralyzing activity and did not cause any observable symptoms after administration of similar doses. On a weight basis, most compounds had a somewhat greater anticonvulsant action than tridione but possessed a somewhat narrower margin between doses causing an elevation of the threshold and those causing generalized symptoms.

DISCUSSION

Paralyzing activity of the type described in the introduction of this paper appears to depend on the structure $R-O-CH_2-CHOH-CH_2OH$ or $R-S-CH_2-CHOH-CH_2OH$. An increase or decrease in the length of this chain caused a decrease or disappearance of paralyzing action as witnessed by the slight activity or inactivity of the glycol, erythritol and mannitol homologues. Substitution in the hydroxyl groups also decreased or destroyed activity. Methyl substitution on the C_2 atom did not materially alter the paralyzing potentiality of the basic structure. These C_2 methyl substituted compounds were much less water soluble but possessed a comparable fat solubility to the unsubstituted straight chain compounds. As both kind of compounds possessed comparable activity

TABLE 8

The anticonvulsant action of 3-aryloxypropan-1,2-diols and 3-arylthioprop-1,2-diols after oral administration to rats convulsed by an interrupted electric current

NO.	R-CH ₂ -CHOH-CH ₂ -OH SUBSTITUENT R	DOSE ORALLY	RESPONSE*	MEAN ELEVATION OF THRESHOLD	PD ₅₀ ± SE IN MICE INTRA- PERITONEALLY	LD ₅₀ ± SE IN MICE INTRA- PERITONEALLY
		mg./kg.		volts	mg./kg.	mg./kg.
1	Phenoxy	400	3/4	75 ± 15	420 ± 14	1240 ± 93
2	2'-methylphenoxy	400	4/4	36 ± 18	180 ± 7	600 ± 16
		200	0/4	0		
3	3'-methylphenoxy	400	4/4	73 ± 2	255 ± 9	750 ± 30
		200	2/4	61 ± 4		
4	4'-methylphenoxy	400	4/4	45 ± 18	340 ± 15	1020 ± 48
		200	2/4	12 ± 2		
5	2'-ethylphenoxy	400	4/1	85 ± 3	255 ± 9	560 ± 15
		200	7/8	35 ± 2		
6	4'-ethylphenoxy	400	4/4	54 ± 18	820† ± 38	1450† ± 67
		200	3/4	17 ± 4		
7	2'-methoxyphenoxy	400	4/4	58 ± 15	365 ± 11	1160 ± 51
		200	2/4	10 ± 2		
8	3'-methoxyphenoxy	400	3/4	50 ± 5	495 ± 41	1350 ± 72
		200	2/4	45 ± 10		
9	4'-methoxyphenoxy	400	3/4	76 ± 19	745 ± 60	1650 ± 99
		200	2/4	55 ± 20		
10	2'-ethoxyphenoxy	400	4/4	90 ± 3	250 ± 7	800 ± 35
		200	4/4	37 ± 14		
11	4'-ethoxyphenoxy	400	4/4	61 ± 25	412 ± 15	740 ± 35
		200	2/4	20 ± 5		
12	2'-propoxyphenoxy	400	4/4	90 ± 5	260 ± 12	650 ± 54
		200	3/4	25 ± 3		
13	2'-butoxyphenoxy	400	4/4	77 ± 10	216 ± 22	710 ± 24
		200	3/4	13 ± 4		
14	2'-heptoxyphenoxy	400	4/4	57 ± 9	>2100†	>2100†
		200	2/4	17 ± 10		
15	2'-alloxyphenoxy	400	4/4	60 ± 14	150 ± 11	650 ± 55
		200	2/4	17 ± 7		
16	Phenylthio	400	4/4	20 ± 10	280 ± 30	940 ± 95
17	2'-methylphenylthio	400	4/4	95 ± 8	210 ± 11	600 ± 30
		200	3/4	63 ± 15		
18	4'-methylphenylthio	400	2/4	60 ± 30	240 ± 15	680 ± 43
19	2'-methoxyphenylthio	400	2/4	60 ± 5	340 ± 26	>940
20	4'-methoxyphenylthio	400	4/4	47 ± 11	510 ± 42	>940
		200	0/4			
21	Tridione	600	4/4	81 ± 8	1750 ± 100	2400 ± 180
		400	1/4	65		
		200	0/4			

* Response $\frac{\text{Number of animals showing an increase in threshold}}{\text{number of animals tested}}$

† Subcutaneous administration in gum acacia.

paralyzing activity. It was more toxic than any of the oxygen ethers and possessed convulsant properties.

II. THE ANTICONVULSANT PROPERTY OF GLYCEROL ETHERS

1) *The antagonism to metrazol.* The effect of compounds No. 1,2,4,5,7 and 9 of table 8 on convulsions produced by metrazol was examined in mice. The compounds were administered intraperitoneally and after a period of 15 minutes metrazol 100 mg. per kg. was injected intravenously. The injection of metrazol was spread over one minute. All control animals treated with metrazol only convulsed and about one half of them died. The various compounds prevented the occurrence of convulsions and death when administered in paralyzing doses, but had little or no effect in smaller doses. Tridione, on the other hand suppressed convulsions in doses of 600 mg. per kg. which was about one third of the dose causing loss of the righting reflex.

2) *The effect of drugs on electrically induced convulsions.* Convulsions were induced in rats by a square wave current of a frequency of 100 cycles per second, administered for 2 seconds. The electrodes were placed at the occiput and in the mouth of the animals. At intervals of 10 or more minutes current of increasing strength was applied until the convulsive threshold was found. The drugs were then administered orally in a suspension of gum acacia and the threshold re-examined at intervals for 3 hours. The highest elevation of threshold obtained with various doses within this period of time is given in table 8. The table also gives the mean paralyzing and lethal doses of the compounds after intraperitoneal administration of aqueous solutions to mice.

All the compounds examined elevated the threshold to electric convulsions to a similar extent and there was no correlation between anticonvulsive and paralytic activities of the substances. For instance, compounds No. 2 and 10, which possessed considerable paralytic activity and caused incoordination and marked depression of spontaneous activity in rats after doses of 400 mg. per kg., did not cause a significantly greater reduction of the irritability of the cortex to electric current than compounds No. 9 and 14 which possessed but little paralyzing activity and did not cause any observable symptoms after administration of similar doses. On a weight basis, most compounds had a somewhat greater anticonvulsant action than tridione but possessed a somewhat narrower margin between doses causing an elevation of the threshold and those causing generalized symptoms.

DISCUSSION

Paralyzing activity of the type described in the introduction of this paper appears to depend on the structure $R-O-CH_2-CHOH-CH_2OH$ or $R-S-CH_2-CHOH-CH_2OH$. An increase or decrease in the length of this chain caused a decrease or disappearance of paralyzing action as witnessed by the slight activity or inactivity of the glycol, erythritol and mannitol homologues. Substitution in the hydroxyl groups also decreased or destroyed activity. Methyl substitution on the C_2 atom did not materially alter the paralyzing potentiality of the basic structure. These C_2 methyl substituted compounds were much less water soluble but possessed a comparable fat solubility to the unsubstituted straight chain compounds. As both kind of compounds possessed comparable activity

if administered in solutions, it may be inferred that paralyzing activity is not correlated with the oil water coefficient.

The radical R in the structure $R-O-CH_2-CHOH-CH_2OH$ could be an acyclic or aromatic hydrocarbon. When R was an aliphatic radical, compounds with straight chain alkyls had greater paralyzing activity than the branched chain isomers. Unsaturated groups decreased activity and increased toxicity.

The greatest paralyzing activity was obtained when R was a benzene ring with a suitable group in ortho position. The presence of a substituent in ortho position was relatively more important than the nature of the substituent. Methyl, ethyl, methoxy and ethoxy groups or a halogen in ortho position produced compounds of marked paralyzing action and a relatively wide margin of safety. The presence of the same groups in para position resulted in markedly less active compounds which were also less toxic and possessed a narrower margin of safety than the ortho isomers. Nuclear substitution with the propoxy group in the ortho position resulted in compounds of marked activity, but further lengthening of the alkoxy side chain reduced activity. Nuclear substitution with a hydroxy, hydroxymethyl, formyl or allyl group destroyed or diminished the paralyzing action of the compounds even when these groups were in ortho position.

Most α substituted ethers of glycerol in toxic doses caused paralysis prior to death. By introduction of a benzene nucleus suitably substituted in ortho position, the paralyzing activity of compounds was increased to a greater extent than their toxicity. The compound of this type with the widest margin of safety and greatest solubility was myanesin, the 3-(2'-methylphenoxy) propan-1,2-diol.

Compounds having the structure $R-O-CH_2-CHOH-CH_2OH$ or $R-S-CH_2-CHOH-CH_2OH$ increased the threshold to electrically induced convulsions. This cortical effect was of approximately the same intensity in all compounds tested and could not be significantly altered by modifications of the radical R. There was no simple correlation between the paralyzing and anticonvulsant properties of the compounds.

α substituted ethers of glycerol possessed also other interesting pharmacological properties. They had a depressant effect on the blood pressure, reduced the rate of the heart, possessed a quinidine-like action on the isolated auricle and were vasodilators. They did not possess central analgesic activity but had an antipyretic action. Numerous compounds possessed local anaesthetic properties and these were particularly well marked in esters of 3-carboxyphenoxypropan-1,2-diol. With the exception of 8-(2',3'-dihydroxypropoxy)-1,1-dimethyl-1,2,3,4-tetrahydroquinolinium iodide none of the compounds possessed curare-like action in tolerated doses. The relaxant and paralyzing effects were caused by the depressant action of the compounds on the central nervous system.

SUMMARY

1) Many substances of the structure $R-O-CH_2-CHOH-CH_2OH$ produced transient muscular relaxation and paralysis. These effects were due to a

depressant action on the central nervous system and particularly on the spinal cord.

2) The paralyzing action was strongest when R was a benzene nucleus substituted in the ortho position with a small alkyl or alkoxy group or chlorine. Compounds with these radicals in meta or para position were less active than the ortho isomers. The presence of a hydroxy, amino, amido, ester or hydroxy-alkyl group or multiple substitution in the ring with alkyls, halogens or both decreased paralyzing activity.

3) When R was an aliphatic radical, straight chain alkyls contributed more to the paralyzing activity than the branched chain isomers or unsaturated radicals. The n-amyl ether was the most potent compound of the aliphatic series.

4) Methyl substitution on the C₂ atom of the glyceryl side chain did not materially alter paralyzing activity but substitution on the C₁ atom decreased activity.

5) Substitution in the hydroxyl groups decreased or destroyed paralyzing activity.

6) Compounds possessing the structure $R-S-CH_2-CHOH-CH_2OH$ or $R-SO_2-CF_2-CHOH-CH_2OH$ also had paralyzing activity, but were more toxic than the oxygen ethers.

7) Compounds of the structure $aryl-O-CH_2-CHOH-CH_2OH$ and $aryl-S-CH_2-CHOH-CH_2OH$ increased the threshold to electrically induced convulsions. There was no simple relation between the anticonvulsant and paralyzing activity of the compounds and the anticonvulsant action could not be significantly altered by alkyl or alkoxy substitution in the nucleus.

My thanks are due to Mr. R. A. Hall and to Misses B. J. O'Brien and D. Culver for technical assistance. Drs. Bradley, Elliott, Forrest, Maclean and Stephenson and Messrs. Baker, Pryce, Sayer and Miss Gailer were responsible for the preparation of the compounds.

REFERENCES

1. GILBERT, A., AND DESCOMPS, P.: C. R. Soc. Biol. Paris, 69: 145, 1910.
2. FOURNEAU, E.: Organic Medicaments and their preparation. Translated by W. A. Silvester, London. 1925.
3. BERGER, F. M., AND BRADLEY, W.: British J. Pharmacol., 1: 265, 1946.
4. BERGER, F. M., AND BRADLEY, W.: Lancet, I: 97, 1947.
5. MALLINSON, F. B.: Lancet, I: 98, 1947.
6. WILSON, H. B., AND GORDON, H. E.: Lancet, I: 367, 1948.
7. STEPHEN, C. R. AND CHANDY, J.: Canad. Med. Ass. J., 57: 463, 1947.
8. SCHLESINGER, E. B., DREW, A. L., AND WOOD, B.: Am. J. Med., 4: 365, 1948.
9. HUNTER, A. R., AND WATERFALL, J. M.: Lancet, I: 366, 1948.
10. BERGER, F. M.: Brit. J. Pharmacol., 2: 241, 1947.
11. BERGER, F. M.: Quart. J. Pharm. Pharmacol., 20: 94, 1947.
12. MILLER, L. C., AND TAINTER, M. L.: Proc. Soc. exp. Biol. and Med., 57: 261, 1944.

THE EFFECTS OF ETHER ON THE MYOCARDIUM OF THE DOG¹

LESLIE L. BENNETT AND CARL W. FISHER

Division of Physiology, The Institute of Experimental Biology, and the Subdivision of Anesthesiology, University of California, Berkeley and San Francisco

Received for publication April 26, 1948

INTRODUCTION

Diethyl ether causes dilatation of the heart in experimental animals by direct action on the heart muscle and at concentrations within the normal anesthetic range. This commonly has been neglected or over-shadowed by discussions of disturbances in rhythm, though earlier workers demonstrated conclusively that such dilatation occurs.

Dieballe (1) and Vernon (2) showed relaxation in amphibian hearts perfused with ether solutions, but stressed the greater toxicity of chloroform. In 1923 Cattell (3) reviewed the literature to that time and did careful experimental studies on the site of action of ether on the circulation in cats, using the pericardial sac as an oncometer. He observed, "From the beginning of the administration of ether, there is relaxation of heart tone as indicated by increased volume. This becomes progressively greater as anesthesia becomes deeper, the maximum dilatation being reached only with the death of the animal." Olmsted and Ogden (4) published a cardiometer tracing made with a dog heart-lung preparation in which 2 or 3 breaths of ether produced marked but transitory increase in ventricular volume. Bhatia and Burn (5) working on dog heart-lung preparations concluded "The administration of ether for quite short periods such as 80 seconds results in an immediate weakening of the power of the heart muscle", and showed similar tracings.

It was proposed to repeat these studies on the dog heart-lung preparation and to correlate the effects with the planes of anesthesia and with the blood ether levels.

METHODS

The heart-lung preparation was used in order to eliminate reflex control of the heart rate and to enable the observations to be carried out under controlled conditions of input-loads and resistance-loads. Aortic pressure was measured and recorded graphically by means of a damped mercury manometer connected to a side arm of the aortic cannula. Right atrial pressure and pulmonary arterial pressure were measured by means of damped water manometers connected with cannulae inserted into the right atrium via the inferior vena cava and into the left upper lobe branch of the pulmonary artery. Ventricular volume was measured by means of a Henderson cardiometer and a piston type volume recorder and was recorded mechanically upon smoked kymograph paper. Left ventricular output minus the coronary flow was measured either by timing outflow into a graduated cylinder with a stopwatch or by a continuously recording siphon-type flow meter.

Blood ether was determined by a slight modification of the Shaffer and Ronzoni (6)

¹ Aided by grants from the Research Board of the University of California.

procedures. Unmedicated dogs were anesthetized with ether and blood samples were taken for ether analysis at the different planes of anesthesia of Stage III. As soon as the head was deprived of its blood supply the anesthetic was discontinued and experimental observations were made about two hours after the last anesthetic was administered. Ether was volatilized and was administered to the heart-lung preparation via the pump that maintained the pulmonary ventilation. At frequent intervals during and after the administration of ether to the heart-lung preparation, arterial blood samples were taken for determination of their ether content. Thus, in each preparation the effects produced upon

TABLE 1

The blood ether level in mgm/100 cc. in normal dogs corresponding to the planes of anesthesia of stage III

	PLANE			
	I	II	III	IV
These experiments	81 \pm 7.0*(13)†	98 \pm 3.4(7)	124 \pm 8.0(7)	154 \pm 8.0(14)
Average of Robbins' (13) observations		103	120	152

* Standard deviation of the mean.

† Number of determinations

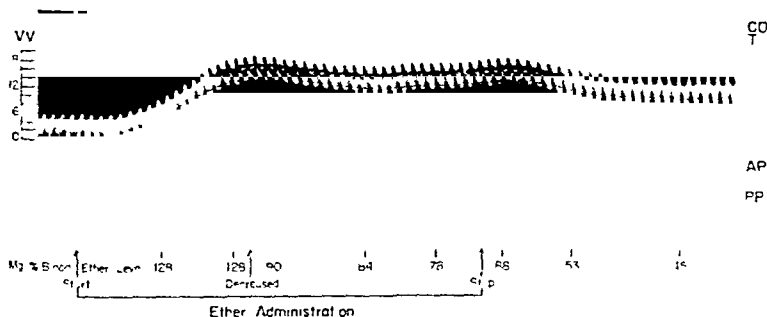


FIG. 1

CO, cardiac output, T, time in minutes, AP, aortic pressure, PP, pulmonary arterial pressure; VV, ventricular volume change calibrated in cc. This experiment illustrates the effects of the administration of ether producing a blood level corresponding to 3rd plane anesthesia and then reduction of its concentration to a level corresponding to 2nd plane anesthesia. Note the ventricular dilatation, with recovery as the blood ether concentration decreased, and the rise in pulmonary pressure (a rise of 25 mm. of H₂O) with subsequent restoration to normal. At the height of the ventricular dilatation right atrial pressure rose 8 mm. of H₂O.

the isolated heart by a given blood ether level could be correlated with the depth of anesthesia previously produced in the intact animal by a comparable level of blood ether. During the course of an experimental observation neither the peripheral resistance nor the venous return were altered.

OBSERVATIONS

Blood ether levels in intact dogs. As reported by Whitehead and Draper (7), marked variations were found in the level of blood ether required to produce the

same plane of anesthesia in different dogs. However, in general the observed levels agreed with those in the literature (8, 9, 10, 11, 12). In table 1 is presented a summary of the blood levels for the planes of Stage III anesthesia. The average levels are in remarkable agreement with those reported by Robbins (13).

The effect of ether upon the heart-lung preparation. In figures 1 and 2 are reproduced representative kymograph records from this series of experiments. In each of the thirteen preparations studied there was definite ventricular dilatation produced by pre-anesthetic blood ether levels, and the dilatation increased progressively with increasing blood ether concentrations. With blood ether levels that corresponded to the 2nd plane of anesthesia there were minimal

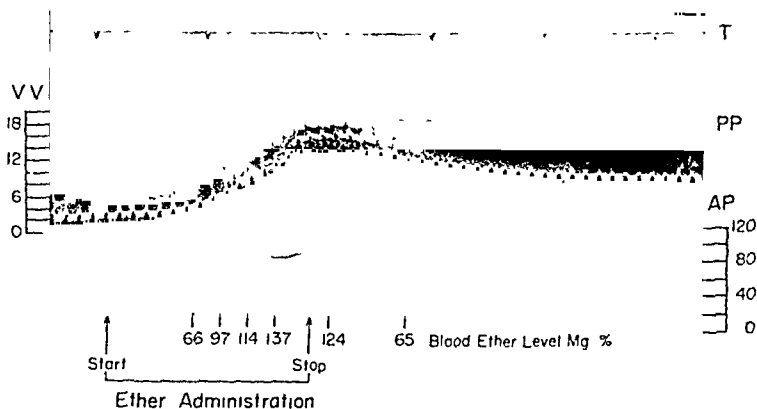


FIG 2

T, time in minutes; PP pulmonary arterial pressure; AP, aortic pressure calibrated in mm of Hg; VV, ventricular volume change calibrated in cc. This experiment illustrates the effects produced by the rapid administration of ether producing a blood level corresponding to 4th plane anesthesia. Note the marked ventricular dilatation with reduction in stroke output and hence cardiac output, the fall in aortic pressure, the rise in pulmonary pressure, and the recovery as the blood ether concentration decreased.

elevations in the pressures in the pulmonary artery and the right atrium. With blood ether levels corresponding to 3rd plane anesthesia these pressure rises were pronounced and were rapidly accentuated when the blood ether concentration was increased to levels corresponding to 4th plane anesthesia.

Only when the ventricular dilatation was marked and there were rises in pulmonary arterial and right atrial pressures did the cardiac output or the aortic pressure decrease. The dynamic changes produced by high blood ether concentrations are characteristic of a failing heart. The fact that low concentrations of blood ether produce ventricular dilatation without change in cardiac output, or change in the aortic, pulmonary arterial or right atrial pressures is good evidence that the effect is produced by a direct impairment of the force of ventricular contraction.

CONCLUSIONS

1. Ether at a blood concentration corresponding to all anesthetic ranges produces ventricular dilatation in the dog heart-lung preparation.
2. Beginning with blood levels corresponding to second plane anesthesia and at all greater concentrations the dynamic changes produced are those of a failing heart.

REFERENCES

1. DIEBALLA, G.: Arch. f. exper. Path. u. Pharmacol., 34: 137, 1894.
2. VERNON, H. M.: J. Physiol., 40: 295, 1910.
3. CATTELL, McK.: Arch. Surg., 6: 41, 1923.
4. OLMSTED, J. M. D., AND OGDEN, E.: Proc. Soc. Exper. Biol. and Med., 27: 725, 1930.
5. BHATIA, B. B., AND BURN, J. H.: J. Physiol., 78: 257, 1933.
6. SHAFFER, P. A., AND RONZONI, E.: J. Biol. Chem., 57: 741, 1923.
7. WHITEHEAD, R. W., AND DRAPER, W. B.: This Journal, 57: 149, 1936.
8. POTTER, R., LIVINGSTONE, H., ANDREWS, E., AND LIGHT, G.: Surgery, 10: 757, 1941.
9. TURPEINEN, E.: Acta chir. Scandinav. (Supp 87), 90: 1, 1942.
10. WEBB, P. K.: Proc. Soc. Exper. Biol. and Med., 23: 75, 1925.
11. RONZONI, E.: J. Biol. Chem., 57: 761, 1923.
12. KRUSE, T. K.: Proc. Soc. Exper. Biol. and Med., 21: 83, 1923.
13. ROBBINS, B. H.: This Journal, 85: 192, 1945.

INDEX

- Adrenalectomy, Effect of, on morphine analgesia in rats, 161
- Adrenergic blocking drugs, Comparisons of effectiveness of, in decreasing epinephrine toxicity in mice, 434
- Alicyclic derivatives of aliphatic amines; Comparison of pressor action of, 423
- Alvarez, Elsie, Whitesell, Marie F., Woodard, Geoffrey, Hagan E. Conway, Nelson, Arthur A., and Draize, John H. Toxicological investigations of compounds proposed for use as insect repellents, 26
- Amphetamine and cocaine, Effects of, on neuromuscular function, 261
- Anderson, Robert C., Worth, Harold M., and Chen, K. K. Ouabagenin, 156
- Anderson, Frederick F., and Craver, Bradford N. Pyrex apparatus for perfusion of coronary circulation of mammalian hearts, 135
- Anderson, Mona M., Parke, Hervey C., and Winder, Claude V. Comparative properties of six phenethylamines, with observations on nature of tachyphylaxis, 63
- Anslow, William P., Jr., Karnofsky, David A., Jager, B. Val, and Smith, Homer W. Toxicity of mustard gas and derivatives, 1
- Anti-histaminic drugs, Effect of, on rabbit myometrium, 196
- Antihistaminics and lysocithin, Inhibition by, of smooth muscle, 457
five, Comparative effectiveness of, vs. histamine-induced spasm in canine Thiry-Vella loops, 163
- Antimalarial drugs, Inhibition by, of cholinesterases of human erythrocytes and plasma, 230
- ANTU toxicity to rats, Effect on, of environmental temperature, 420
toxicity to rats, Effect on, of various sulfur-containing compounds, 414
- Bartlett, Grant R. Inhibition of succinoxidase by vitamin P-like flavonoid 2',3,4-trihydroxy chalcone, 329
- Belkin, Morris. Effect of podophyllin on transplanted mouse tumors, 18
- Bennett, Leslie L., and Fisher, Carl W. Effects of ether on myocardium of dog, 482
- Benzene hexachloride, gamma isomer of, Tissue distribution of toxicant following oral ingestion of, by rats, 277
- Beralds, Wilson T., and Rocha e Silva, M. Dynamics of recovery and measure of drug antagonism. Inhibition of smooth muscle by lysocithin and antihistaminics, 457
- Bergen, F. M. Relationship between chemical structure and central depressant action of α substituted ethers of glycerol, 470
and Schwartz, R. P. Toxicity and muscular effect of d-tubocurarine combined with β -erythroidine, myanesin or evipal, 362
- Beyer, Karl H., Latven, Albert R., Freyburger, Walter A., and Parker, Mary P. Comparative study of activity and toxicity of hexylcaine, 388
- Bird, Joseph G., Cook, Sally, Krantz, John C., Jr., and Carr, C. Jelleff. Pharmacodynamic studies of polyoxyalkylene derivatives of hexitol anhydride partial fatty acid esters, 188
- Blood, rat, coagulation time of, Effect on, of rutin, 333
sugar and rectal temperature of rabbits, Effects on, of corn oil and olive oil, 346
- Braun, Herbert A., and Lusky, Lehman M. Comparative study of intravenous pigeon and intravenous cat method in assay of digitalis, 81
- Brown, George E., Jr., Wood, Earl H., and Lambert, Edward H. Effects of intravenous injection of tetra-ethylammonium chloride on intra-arterial blood pressure and other physiologic variables in man, 10
- Buechler, Edith, and Guggenheim, K. Effect of quantitative and qualitative protein deficiency on tolerance to emetine, 273
- Cameron, Anne, Yonkman, Fredrick F., and Craver, Bradford N. Comparative effectiveness of five antihistaminics

- vs. histamine-induced spasm in canine Thiry-Vella loops, 168
- Carbohydrate metabolism, Effect on, of DTB, 93
- Carr, C. Jelleff, Bird, Joseph G., Cook, Sally, and Krantz, John C., Jr. Pharmacodynamic studies of polyoxyalkylene derivatives of hexitol anhydride partial fatty acid esters, 188
- Chen, Graham, and Clarke, Isabel G. Influence of diphenhydramine hydrochloride and epinephrine on glucose metabolism in rabbit, 175
- Chen, K. K., and Swanson, Edward E. Comparison of pressor action of alicyclic derivatives of aliphatic amines, 423
- Anderson, Robert C., and Worth, Harold M. Ouabagenin, 156
- Scott, C. C., and Robbins, E. B. Pharmacologic comparison of optical isomers of methadon, 282
- Chloroquine, Chronic oral toxicity of, 147
- Cholinesterases of human erythrocytes and plasma and their inhibition by antimalarial drugs, 230
- Chondrodendrine derivatives, Curariform activity of, 109
- Clarke, Isabel G., and Chen, Graham. Influence of diphenhydramine hydrochloride and epinephrine on glucose metabolism in rabbit, 175
- Cocaine and amphetamine, effects of, on neuromuscular function, 261
- Cook, Sally, Krantz, John C., Jr., Carr, C. Jelleff, and Bird, Joseph G. Pharmacodynamic studies of polyoxyalkylene derivatives of hexitol anhydride partial fatty acid esters, 188
- Coronary circulation of mammalian hearts, Pyrex apparatus for perfusion of, 135
- Craver, Bradford N., and Anderson, Frederick F. Pyrex apparatus for perfusion of coronary circulation of mammalian hearts, 135
- Cameron, Anne, and Yonkman, Frederick F. Comparative effectiveness of five antihistaminics vs. histamine-induced spasm in canine Thiry-Vella loops, 168
- Cyclohexylalkylamines, Comparative pharmacology of, 338
- DDT, Effect of, on blood sugar and of glucose administration on acute and chronic DDT poisoning in rabbits, 351
- Depressants of central nervous system, Effect of, on emptying time of human stomach, 208
- Diatrin, Toxicologic and antihistaminic properties of, 210
- Dibenamine, Locus of adrenergic blocking action of, 40
- Digitalis, assay of, Comparative study of intravenous pigeon and intravenous cat method in, 81
- Digitalis-like Colombian arrow poison: niaara, 223
- Diphenhydramine hydrochloride and epinephrine, Influence of, on glucose metabolism in rabbit, 175
- Dithiobiuret (DTB), Effect of, on electrolyte and water content of skeletal muscle and on carbohydrate metabolism, 93
- Dithiocarbamates and their bismuth derivatives, Chemotherapeutic studies on a series of, 430
- Draize, John H., Alvarez, Elsie, Whitesell, Marie F., Woodard, Geoffrey, Hagan, E. Conway, and Nelson, Arthur A. Toxicological investigations of compounds proposed for use as insect repellents, 26
- Drug antagonism, Measure of, and dynamics of recovery, 457
- Eisenman, Anna J., and Isbell, Harris. Addiction liability of some drugs of methadon series, 305
- Emetine, tolerance to, Effect on, of quantitative and qualitative protein deficiency, 273
- Epinephrine and diphenhydramine hydrochloride, Influence of, on glucose metabolism in rabbit, 175
- toxicity in mice, Comparisons of effectiveness of adrenergic blocking drugs in decreasing, 434
- Ercoli, N., Schachter, R. J., Hueper, W. C., and Lewis, M. N. Toxicologic and antihistaminic properties of diatrin, 210
- Ether, Effects of, on myocardium of dog, 482
- Eyzaguirre, C., Perez, F., and Luco, J. V. Effects of amphetamine and cocaine on neuromuscular function, 261
- "Fanyline", Rodenticidal activity of, and its oral toxicity to several species, 287

- Fisher, Carl W., and Bennett, Leslie L. Effects of ether on myocardium of dog, 482
- Fitzhugh, O. Garth, Nelson, Arthur A., and Holland, Oma L. Chronic oral toxicity of chloroquine, 147
- Flavonoid 2',3,4-trihydroxy chalcone, vitamin P-like, Inhibition by, of succinoxidase, 329
- Fluharty, R. G., Wilson, Mary, Riddell, Charlotte, Yee, Hilda, McGinty, D. A., and Rawson, R. W. Effect of certain goitrogenic drugs on absorption of radioactive iodine by thyroid gland, 246
- Foa, Piero P., Weinstein, Harriet R., Ludwig, A. S., Wertheim, J. Marvin, and Smith, Jay A. Some aspects of thiamine toxicity, 294
- Freyburger, Walter A., Parker, Mary P., Beyer, Karl H., and Latven, Albert R. Comparative study of activity and toxicity of hexylcaine, 388
- Friend, Frances J., and Harris, S. C. Effect of adrenalectomy on morphine analgesia in rats, 161
- Ganglia, sympathetic, Blocking action of magnesium ion on, 52
- Glucose administration, Effect of, on acute and chronic DDT poisoning in rabbits, 351
- metabolism in rabbit, Influence on, of diphenhydramine hydrochloride and epinephrine, 175
- Glycerol, α substituted ethers of, Relationship between chemical structure and central depressant action of, 470
- Goitrogenic drugs, Effect of certain, on absorption of radioactive iodine by thyroid gland of rats and chicks, 240, 246
- Goth, Andres, and Robinson, Fabian J. Chemotherapeutic studies on a series of dithiocarbamates and their bismuth derivatives, 430
- Gruber, Charles M., Halbeisen, William A., and Gruber, Charles M., Jr. Toxicologic and pharmacologic investigation of medomin, 101
- Gruber, Charles M., Jr., Gruber, Charles M., and Halbeisen, William A. Toxicologic and pharmacologic investigation of medomin, 101
- Guggenheim, K., and Buechler, Edith. Effect of quantitative and qualitative protein deficiency on tolerance to emetine, 273
- Hagan, E. Conway, Nelson, Arthur A., Draize, John H., Alvarez, Elsie, Whitesell, Marie F., and Woodard, Geoffrey. Toxicological investigations of compounds proposed for use as insect repellents, 26
- Halbeisen, William A., Gruber, Charles M., Jr., and Gruber, Charles M. Toxicologic and pharmacologic investigation of medomin, 101
- Harkness, David M., Muntwyler, Edward, Seifter, Joseph, and Seifter, Sam. Effect of DTB on electrolyte and water content of skeletal muscle and on carbohydrate metabolism, 93
- Harris, S. C., and Friend, Frances J. Effect of adrenalectomy on morphine analgesia in rats, 161
- Heal, Ralph E., and Kuna, Samuel. Toxicological and pharmacological studies on powdered stem of *Ryania speciosa*, 407
- Heideman, M. Lawrence, Jr., and Rutledge, Robert C., Jr. Determination of sulfamylon in biological fluids, 451
- Hexitol anhydride partial fatty acid esters, Pharmacodynamic studies of polyoxyalkylene derivatives of, 188
- Hexylcaine, Comparative study of activity and toxicity of, 388
- Histamine-induced spasm in canine Thiry-Vella loops, Comparative effectiveness of five antihistaminics vs., 168
- Holland, Oma L., Fitzhugh, O. Garth, and Nelson, Arthur A. Chronic oral toxicity of chloroquine, 147
- Howie, Donald L., Weiss, Rowena, Swanson, Rollan, Way, E. Leong, and Smith, Paul K. Absorption, distribution, excretion and fate of para-aminosalicylic acid, 368
- Hueper, W. C., Lewis, M. N., Ercoli, N., and Schachter, R. J. Toxicologic and antihistaminic properties of diatrin, 210
- Huertas-Lozano, J., Mezey, K., Uribe-Piedrahita, C., and Pataki, J. Niaara, 223
- Hunt, Reid, 1870-1948, 259
- Iodine, radioactive, absorption by thyroid gland of rats and chicks, Effect on, of certain goitrogenic drugs, 240, 246

- Insect repellents, Toxicological investigations of compounds proposed for use as, 26
- Isbell, Harris, and Eisenman, Anna J. Addiction liability of some drugs of methadon series, 305
- Jager, B. Val, Smith, Homer W., Anslow, William P., Jr., and Karnofsky, David A. Toxicity of mustard gas and derivatives, 1
- Kaiser, Irwin H., and Reynolds, S. R. M. Quantitative measurement of uterine responses using strain gage dynamometer, with notes on effect of antihistaminic drugs on rabbit myometrium, 196
- Kaiser, Margaret E., and Winder, Claude V. Relative experimental coronary vasodilator potencies and toxicities of papaverine, theophylline, and a papaverine, theophylline, molecular association compound, 86
- Karel, Leonard. Rodenticidal activity of "Fanyline" and its oral toxicity to several species, 287
- and Meyer, Bertram J. Effect of environmental temperature on ANTU toxicity to rats, 420
- and Meyer, Bertram J. Effect of various sulfur-containing compounds on ANTU toxicity to rats, 414
- Karnofsky, David A., Jager, B. Val, Smith, Homer W., and Anslow, William P., Jr. Toxicity of mustard gas and derivatives, 1
- Krantz, John C., Jr., Carr, C. Jelleff, Bird, Joseph G., and Cook, Sally. Pharmacodynamic studies of polyoxyalkylene derivatives of hexitol anhydride partial fatty acid esters, 188
- Kuna, Samuel, and Heal, Ralph E. Toxicological and pharmacological studies on powdered stem of *Ryania speciosa*, 407
- Lambert, Edward H., Brown, George E., Jr., and Wood, Earl H. Effects of intravenous injection of tetra-ethylammonium chloride on intra-arterial blood pressure and other physiologic variables in man, 10
- Latven, Albert R., Freyburger, Walter A., Parker, Mary P., and Beyer, Karl H. Comparative study of activity and toxicity of hexylcaine, 388
- Laug, Edwin P. Tissue distribution of toxicant following oral ingestion of gamma isomer of benzene hexachloride by rats, 277
- Lehmann, G., and Randall, Lowell O. Pharmacological properties of sympathomimetic diamines, 114
- and Randall, Lowell O. Pharmacological studies on analgesic piperidine derivatives, 314
- Leonards, Jack R., and Williams, Florence. Effect of sodium bicarbonate on renal excretion of salicylate, 401
- Leow, Earl R., and Micetich, Audrey. Comparisons of effectiveness of adrenergic blocking drugs in decreasing epinephrine toxicity in mice, 434
- Lewis, M. N., Ercoli, N., Schachter, R. J., and Hueper, W. C. Toxicologic and antihistaminic properties of diatrin, 210
- Lillie, R. D., and Stohlman, E. F. Effect of DDT on blood sugar and of glucose administration on acute and chronic DDT poisoning in rabbits, 351
- Lockhart, Helen, Rawson, R. W., McGinty, D. A., Peacock, Wendell, Merrill, Priscilla, and Wilson, Mary. Effect of certain goitrogenic drugs on absorption of radioactive iodine by thyroid gland of rats and chicks, 240
- Luco, J. V., Eyzaguirre, C., and Perez, F. Effects of amphetamine and cocaine on neuromuscular function, 261
- Ludwig, A. S., Wertheim, J. Marvin, Smith, Jay A., Foa, Piero P., and Weinstein, Harriet R. Some aspects of thiamine toxicity, 294
- Lusky, Lehman M., and Braun, Herbert A. Comparative study of intravenous pigeon and intravenous cat method in assay of digitalis, 81
- Lysocithin and antihistaminics, Inhibition by, of smooth muscle, 457
- Magnesium ion, Blocking action of, on sympathetic ganglia, 52
- Marsh, David F. Comparative pharmacology of cyclohexylalkylamines, 338
- Sleeth, Clark K., and Tucker, Eldon B. Curariform activity of d-N-methyl-

- chondrodendrine and d-O-methyl-N-methyl-chondrodendrine, 109
- McGinty, D. A., Peacock, Wendell, Merrill, Priscilla, Wilson, Mary, Lockhart, Helen, and Rawson, R. W. Effect of certain goitrogenic drugs on absorption of radioactive iodine by thyroid gland of rats and chicks, 240
- Rawson, R. W., Fluharty, R. G., Wilson, Mary, Riddell, Charlotte, and Yee, Hilda. Effect of certain goitrogenic drugs on absorption of radioactive iodine by thyroid gland, 246
- Medomin (cycloheptenylethyl barbituric acid), Toxicologic and pharmacologic investigation of, 101
- Merrill, Priscilla, Wilson, Mary, Lockhart, Helen, Rawson, R. W., McGinty, D. A., and Peacock, Wendell. Effect of certain goitrogenic drugs on absorption of radioactive iodine by thyroid gland of rats and chicks, 240
- Methadon, Pharmacologic comparison of optical isomers of, 282
- series, some drugs of, Addiction liability of, 305
- Meyer, Bertram J., and Karel, Leonard. Effect of environmental temperature on ANTU toxicity to rats, 420
- and Karel, Leonard. Effect of various sulfur-containing compounds on ANTU toxicity to rats, 414
- Mezey, K., Uribe-Predrahitia, C., Pataki, J., and Huertas-Lozano, J. Niaara, 223
- Micetich, Audrey, and Leow, Earl R. Comparisons of effectiveness of adrenergic blocking drugs in decreasing epinephrine toxicity in mice, 434
- Money, W. L., and Zarrow, M. X. Pharmacology of relaxin, 180
- Morphine analgesia in rats, Effect on, of adrenalectomy 161
- Munch, James C., Wolffe, J. B., and Plungian, M. B. Effect of rutin on coagulation time of rat blood, 383
- Muntwyler, Edward, Seifter, Joseph, Seifter, Sam, and Harkness, David M. Effect of DTB on electrolyte and water content of skeletal muscle and on carbohydrate metabolism, 93
- Muscle, skeletal, electrolyte and water content of, Effect on, of DTB, 93
- Mustard gas and derivatives, Toxicity of, 1
- Myanesin, Determination of, in body fluids and tissues, and its disappearance from blood after intravenous injections in dog, 129
- Myocardium of dog, Effects on, of ether, 482
- Myometrium, rabbit, Effect on, of anti-histaminic drugs, 196
- Nelson, Arthur A., Draize, John H., Alvarez, Elsie, Whitesell, Marie F., Woodard, Geoffrey, and Hagan, E. Conway. Toxicological investigations of compounds proposed for use as insect repellents, 26
- Holland, Oma L., and Fitzhugh, O. Garth. Chronic oral toxicity of chloroquine, 147
- Niaara, a digitalis-like Colombian arrow poison, 223
- Nickerson, Mark, and Nomaguchi, George M. Locus of adrenergic blocking action of dibenamine, 40
- Nitrite-producing systems in rabbit tissues, 444
- Nomaguchi, George M., and Nickerson, Mark. Locus of adrenergic blocking action of dibenamine, 40
- Northup, David W., and Van Liere, Edward J. Effect of central nervous system depressants on emptying time of human stomach, 208
- Oberst, Fred W., and Snyder, Fred H. Nitrite-producing systems in rabbit tissues, 444
- Oil, corn and olive, Effects of, on blood sugar and rectal temperature of rabbits, 346
- Ouabagenin, 156
- Papaverine, theophylline, and apapaverine-theophylline, molecular association compound, Relative experimental coronary potencies and toxicities of, 86
- Para-aminosalicylic acid, Absorption, distribution, excretion and fate of, 368
- Parke, Hervey C., Winder, Claude V., and Anderson, Mona M. Comparative properties of six phenethylamines, with observations on nature of tachyphylaxis, 63
- Parker, Mary P., Beyer, Karl H., Latven, Albert R., and Freyburger, Walter A.

- Comparative study of activity and toxicity of hexylcaine, 388
- Pataki, J., Huertas-Lozano, J., Mezey, K., and Uribe-Piedrahita, C. Naaara, 223
- Peacock, Wendell, Merrill, Priscilla, Wilson, Mary, Lockhart, Helen, Rawson, R. W., and McGinty, D. A. Effect of certain goitrogenic drugs on absorption of radioactive iodine by thyroid gland of rats and chicks, 240
- Perez, F., Luco, J. V., and Eyzaguirre, C. Effects of amphetamine and cocaine on neuromuscular function, 261
- Phenethylamines, six, Comparative properties of, with observations on nature of tachyphylaxis, 63
- Piperidine derivatives, analgesic, Pharmacological studies on, 314
- Plummer, Albert J. Method for quantitative estimation of theophylline in blood and urine: application to dog, 142
- Plumjian, M. B., Munch, James C., and Wolfe, J. B. Effect of rutin on coagulation time of rat blood, 383
- Podophyllin, Effect of, on transplanted mouse tumors, 18
- Poe, Charles F., and Suchy, John F. Effect of vitamin A deficiency on toxicity of strychnine, 153
- Pressor action, Comparison of, of alicyclic derivatives of aliphatic amines, 423
- Protein deficiency, quantitative and qualitative, Effect of, on tolerance to emetine, 273
- Randall, Lowell O., and Lehmann, G. Pharmacological properties of sympathomimetic diamines, 114
- and Lehmann, G. Pharmacological studies on analgesic piperidine derivatives, 314
- Rawson, R. W., Fluharty, R. G., Wilson, Mary, Riddell, Charlotte, Yee, Hilda, and McGinty, D. A. Effect of certain goitrogenic drugs on absorption of radioactive iodine by thyroid gland, 246
- McGinty, D. A., Peacock, Wendell, Merrill, Priscilla, Wilson, Mary, and Lockhart, Helen. Effect of certain goitrogenic drugs on absorption of radioactive iodine by thyroid gland of rats and chicks, 240
- Relaxin, Pharmacology of, 180
- Reynolds, S. R. M., and Kaiser, Irwin H. Quantitative measurement of uterine responses using strain gage dynamometer, with notes on effect of anti-histaminic drugs on rabbit myometrium, 196
- Richardson, Arthur P., Titus, Elwood, and Ulick, Stanley. Determination of myanesis in body fluids and tissues, and its disappearance from blood after intravenous injections in dog, 129
- Riddell, Charlotte, Yee, Hilda, McGinty, D. A., Rawson, R. W., Fluharty, O. R. G., and Wilson, Mary. Effect of certain goitrogenic drugs on absorption of radioactive iodine by thyroid gland, 246
- Robbins, E. B., Chen, K. K., and Scott, C. C. Pharmacologic comparison of optical isomers of methadon, 282
- Robinson, Fabian J., and Goth, Andres. Chemotherapeutic studies on a series of dithiocarbamates and their bismuth derivatives, 430
- Rocha e Silva, M., and Beraldo, Wilson T. Dynamics of recovery and measure of drug antagonism. Inhibition of smooth muscle by lysocithin and antihistaminics, 457
- Rutin, Effect of, on coagulation time of rat blood, 383
- Rutledge, Robert C., Jr., and Heideman, M. Lawrence, Jr. Determination of sulfamylon in biological fluids, 451
- Ryania speciosa*, Toxicological and pharmacological studies on powdered stem of, 407
- Sabine, Jean C., and Wright, C. I. Cholinesterases of human erythrocytes and plasma and their inhibition by antimalarial drugs, 230
- Salicylate, renal excretion of, Effect on, of sodium bicarbonate, 401
- Schachter, R. J., Hueper, W. C., Lewis, M. N., and Ercoli, N. Toxicologic and antihistaminic properties of diatrin, 210
- Schwartz, R. P., and Berger, F. M. Toxicity and muscular effect of d-tubocurarine combined with β -erythroidine, myaresin or evipal, 362
- Scott, C. C., Robbins, E. B., and Chen, K. K. Pharmacologic comparison of optical isomers of methadon, 282
- Seifter, Joseph, Seifter, Sam, Harkness,

- David M., and Muntwyler, Edward. Effect of DTB on electrolyte and water content of skeletal muscle and on carbohydrate metabolism, 93
- Seifter, Sam, Harkness, David M., Muntwyler, Edward, and Seifter, Joseph. Effect of DTB on electrolyte and water content of skeletal muscle and on carbohydrate metabolism, 93
- Sleeth, Clark K., Tucker, Eldon B., and Marsh, David F. Curariform activity of d-N-methyl-chondrodendrine and d-d-O-methyl - N-methyl-chondrodendrine, 109
- Smith, Homer W., Anslow, William P., Jr., Karnofsky, David A., and Jager, B. Val. Toxicity of mustard gas and derivatives, 1
- Smith, Jay A., Foa, Piero P., Weinstein, Harriet R., Ludwig, A. S., and Wertheim, J. Marvin. Some aspects of thiamine toxicity, 294
- Smith, Paul K., Howie, Donald L., Weiss, Rowena, Swanson, Rollan, and Way, E. Leong. Absorption, distribution, excretion and fate of para-aminosalicylic acid, 368
- Snyder, Fred H., and Oberst, Fred W. Nitrite-producing systems in rabbit tissues, 444
- Sodium bicarbonate, effect of, on renal excretion of salicylate, 401
- Stanbury, John B. Blocking action of magnesium ion on sympathetic ganglia, 52
- Stohlgman, E. F. Effects of corn oil and olive oil on blood sugar and rectal temperature of rabbits, 346
- and Lillie, R. D. Effect of DDT on blood sugar and of glucose administration on acute and chronic DDT poisoning in rabbits, 351
- Stomach, human, emptying time of, Effect on, of central nervous system depressants, 208
- Strychnine, toxicity of, Effect on, of vitamin A deficiency, 153
- Succinoxidase, Inhibition of, by vitamin P-like flavonoid 2',3,4-trihydroxy chalcone, 329
- Suchy, John F., and Poe, Charles F. Effect of vitamin A deficiency on toxicity of strychnine, 153
- Sulfamylon, Determination of, in biological fluids, 451
- Sulfur-containing compounds, Effect of various, on ANTU toxicity to rats, 414
- Swanson, Edward E., and Chen, K. K. Comparison of pressor action of alicyclic derivatives of aliphatic amines, 423
- Swanson, Rollan, Way, W. Leong, Smith, Paul K., Howie, Donald L., and Weiss, Rowena. Absorption, distribution, excretion and fate of para-aminosalicylic acid, 368
- Sympathomimetic diamines, Pharmacological properties of, 114
- Tachyphylaxis, Observations on nature of, 63
- Tetra-ethyl-ammonium chloride, Effects of intravenous injection of, on intra-arterial blood pressure and other physiologic variables in man, 10
- Theophylline in blood and urine, Method for quantitative estimation of, 142
- papaverine, and a papaverine-theophylline molecular association compound, Rel-experimental coronary potencies and toxicities of, 86
- Thiamine toxicity, Some aspects of, 294
- Thyroid gland of rats and chicks, absorption by, of radioactive iodine, Effect on, of certain goitrogenic drugs, 240, 246
- Titus, Elwood, Ulick, Stanley, and Richardson, Arthur P. Determination of myanesis in body fluids and tissues, and its disappearance from blood following intravenous injections in dog, 129
- d-Tubocurarine combined with β -erythroidine, myanesis or evipal, Toxicity and muscular effect of, 362
- Tucker, Eldon B., Marsh, David F., and Sleeth, Clark K. Curariform activity of d-N-methyl-chondrodendrine and d-O-methyl-N-methyl-chondrodendrine, 109
- Tumors, transplanted mouse, Effect on, of podophyllin, 18
- Ulick, Stanley, Richardson, Arthur P., and Titus, Elwood. Determination of myanesis in body fluids and tissues, and its disappearance from blood following intravenous injections in dog, 129
- Uribe-Piedrahita, C., Pataki, J., Huertas-Lozano, J., and Mezey, K. Niarara, 223
- Uterine responses, quantitative measure-

- ment of, using strain gage dynamometer, 196
- Van Liere, Edward J., and Northup, David W. Effect of central nervous system depressants on emptying time of human stomach, 208
- Vitamin A deficiency, Effect of, on toxicity of strychnine, 153
- Wallace, George Barclay, 1874-1948, 127
- Way, E. Leong, Smith, Paul K., Howie, Donald L., Weiss, Rowena and Swanson, Rollan. Absorption, distribution, excretion and fate of para-aminosalicylic acid, 368
- Weinstein, Harriet R., Ludwig, A. S., Wertheim, J. Marvin, Smith, Jay A., and Foa, Piero P. Some aspects of thiamine toxicity, 294
- Weiss, Rowena, Swanson, Rollan, Way, E. Leong, Smith, Paul K., and Howie, Donald L. Absorption, distribution, excretion and fate of para-aminosalicylic acid, 368
- Wertheim, J. Marvin, Smith, Jay A., Foa, Piero P., Weinstein, Harriet R., and Ludwig, A. S. Some aspects of thiamine toxicity, 294
- Whitesell, Marie F., Woodard, Geoffrey, Hagan, E. Conway, Nelson, Arthur A., Draize, John H., and Alvarez, Elsie. Toxicological investigations of compounds proposed for use as insect repellents, 26
- Williams, Florence, and Leonards, Jack R. Effect of sodium bicarbonate on renal excretion of salicylate, 401
- Wilson, Mary, Lockhart, Helen, Rawson, R. W., McGinty, D. A., Peacock, Wendell, and Merrill, Priscilla. Effect of certain goitrogenic drugs on absorption of radioactive iodine by thyroid gland of rats and chicks, 240
- Riddell, Charlotte, Yee, Hilda, McGinty, D. A., Rawson, R. W., and Fluharty, R. G. Effect of certain goitrogenic drugs on absorption of radioactive iodine by thyroid gland, 246
- Winder, Claude V., and Kaiser, Margaret E. Relative experimental coronary vasodilator potencies and toxicities of papaverine, theophylline, and a papaverine, theophylline molecular association compound, 86
- Anderson, Mona M., and Parke, Harvey C. Comparative properties of six phenethylamines, with observations on nature of tachyphylaxis, 63
- Wolfe, J. B., Plungian, M. B., and Munch, James C. Effect of rutin on coagulation time of rat blood, 383
- Wood, Earl H., Lambert, Edward H., and Brown, George E., Jr. Effects of intravenous injection of tetra-ethyl-ammonium chloride on intra-arterial blood pressure and other physiologic variables in man, 10
- Woodard, Geoffrey, Hagan, E. Conway, Nelson, Arthur A., Draize, John H., Alvarez, Elsie, and Whitesell, Marie F. Toxicological investigations of compounds proposed for use as insect repellents, 26
- Worth, Harold M., Chen, K. K., and Anderson, Robert C. Ouabagenin, 156
- Wright, C. I., and Sabine, Jean C. Cholinesterases of human erythrocytes and plasma and their inhibition by antimalarial drugs, 230
- Yee, Hilda, McGinty, D. A., Rawson, R. W., Fluharty, R. G., Wilson, Mary, and Riddell, Charlotte. Effect of certain goitrogenic drugs on absorption of radioactive iodine by thyroid gland, 246
- Yonkman, Fredrick F., Craver, Bradford N., and Cameron, Anne. Comparative effectiveness of five antihistaminics vs. histamine-induced spasm in canine Thiry-Vella loops, 168
- Zarrow, M. X., and Money, W. L. Pharmacology of relaxin, 180

